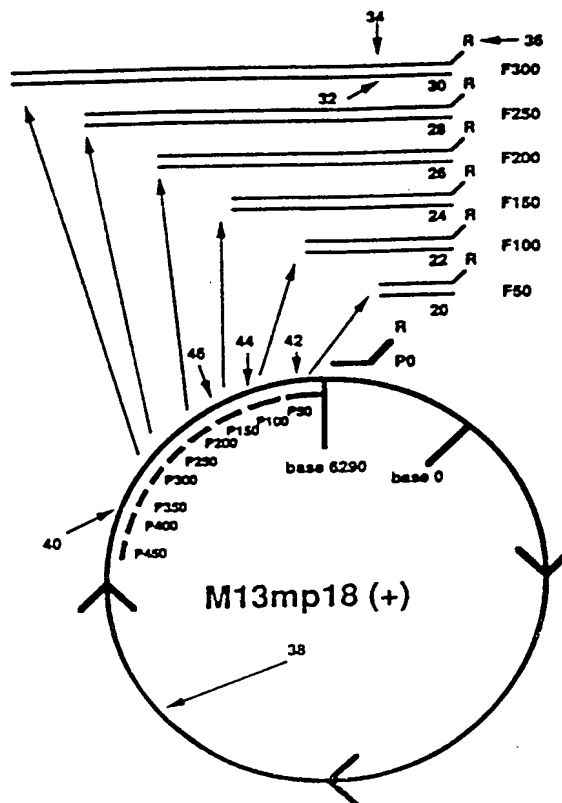


INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵: C12Q 1/68, G01N 27/26	A1	(11) International Publication Number: WO 93/14224 (43) International Publication Date: 22 July 1993 (22.07.93)
(21) International Application Number: PCT/US93/00100 (22) International Filing Date: 6 January 1993 (06.01.93) (30) Priority data: 820,567 14 January 1992 (14.01.92) US (71) Applicant: APPLIED BIOSYSTEMS, INC. [US/US]; 850 Lincoln Centre Drive, Foster City, CA 94404 (US). (72) Inventors: MOON, Richard, B. ; 1339 Pebble Drive, San Carlos, CA 94070 (US). MADONIK, Alex, M. ; 1423 Berkeley Way, Berkeley, CA 94702 (US). RAFF, Mal- colm, I. ; 2735 Ellsworth Street, Berkeley, CA 94705 (US).		(74) Agent: DEHLINGER, Peter, J.; Law Offices of Peter J. Dehlinger, P.O. Box 60850, Palo Alto, CA 94306-0850 (US). (81) Designated States: JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>

(54) Title: SIZE-CALIBRATION DNA FRAGMENT MIXTURE AND METHOD**(57) Abstract**

A size-calibration mixture for use in achieving single basepair resolution of polynucleotide fragments fractionated by high-resolution electrophoresis. The mixture includes two or more polynucleotide strand pairs, each pair having one strand which has a selected number of polynucleotide bases and a detectable reporter in its 5'-end region and a complementary strand which lacks the reporter. Also disclosed are methods of producing and using the mixture.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SK	Slovak Republic
CI	Côte d'Ivoire	LI	Liechtenstein	SN	Senegal
CM	Cameroon	LK	Sri Lanka	SU	Soviet Union
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	MC	Monaco	TC	Togo
DE	Germany	MG	Madagascar	UA	Ukraine
DK	Denmark	ML	Mali	US	United States of America
ES	Spain	MN	Mongolia	VN	Viet Nam
FI	Finland				

5 SIZE-CALIBRATION DNA FRAGMENT MIXTURE AND METHOD1. Field of the Invention

 The present invention relates to a size calibration
10 mixture for use in calibrating the sizes, i.e., number of
 bases, of polynucleotides fractionated by high-resolution
 electrophoresis, and to methods for making and using the
 mixture.

15 2. References

- Cantor, C., et al., "Biophysical Chemistry Parts I, II
and III," W.H. Freeman and Co., N.Y. (1980).
 Carrano, A.V., et al, Genomics, 4:129 (1989).
 Connell, C., et al., BioTechniques, 5:342-348 (1987).
20 Fung, S., et al., U.S. Patent 4,855,225 (1989).
 Litt, M., et al., Am. J. Human Genetics, 44:397-401
 (1989).
 Maniatis, T., et. al., "Molecular Cloning: A Labora-
tory Manual," Cold Spring Harbor Labs (1982).
25 Maxam, A.M., et al., Meth Enzymol, 65:499 (1980).
 Sanger, F., et al., Proc Nat Acad Sci, USA, 74:5363
 (1977).
 Smith, L.M., et al., Nature, 321:674 (1986).
 Weber, J.L., et al., Am. J. Human Genetics, 44:388-396
30 (1989).

3. Background of the Invention

 Methods for size fractionating mixtures of single-
35 strand or double-strand (duplex) nucleic acids are crucial
 to a variety of analytical and preparative techniques in

biochemistry. Such methods generally employ a solid or semi-solid gel matrix for fragment separation. In the case of larger molecular weight fragments, typically greater than about 700 bases, the preferred gel material is agarose, where the concentration of the agarose may vary from about 0.3%, for separating fragments in the 5-60 kilobase size range, up to about 2%, for separating fragments in the 100-3,000 basepair range (Maniatis). Smaller size fragments, less than about 1,200 basepairs, are usually separated in polyacrylamide gel. The concentration of acrylamide polymer can range from about 3.5%, for separating fragments in the larger basepair range, up to about 20%, for achieving separation in the size range 10-100 basepairs.

One important application of DNA size fractionation is restriction-site analysis, in which duplex DNA is digested with selected restriction enzyme(s), fractionated according to digest fragment size, then analyzed for fragment positions. This method is widely used in molecular cloning to determine the number and arrangement of restriction sites in a cloning vector, and to confirm insert location and/or orientation in the vector.

Restriction analysis is an important tool for genetic mapping as well. In one method which has been proposed for human genomic mapping, genomic fragments from a cosmid library are digested with a selected group of restriction enzymes to generate duplex subfragments predominantly less than about 700 basepairs in size. The digest subfragments are then fractionated by polyacrylamide gel, and the subfragment sizes are compared with those of subfragments derived from other library inserts, to identify fragment overlap on the basis of common subfragment sizes. This mapping method can be adapted for subfragment detection by

fluorescent labels, allowing rapid gel fractionation analysis (e.g., Carrano).

The success of the above mapping method depends in part on accurate size determination of the fractionated subfragments. In particular, it is desirable to determine
5 fragment sizes to a resolution of one nucleotide base.

In a related application, the discovery of linkages between a number of genetic diseases and restriction fragment length polymorphisms in humans has provided a tool for
10 screening individuals for these genetic diseases. More recently, genomic fragments containing a variable number of tandem repeat (VNTR) sequences have been discovered, and it is likely that individual-specific VNTR regions may be used as linkage markers for certain genetic diseases. Several
15 VNTR loci which have been reported to date contain a 2-nucleotide repeat sequence (Weber; Litt). Thus, distinguishing VNTRs which differ by a single repeat sequence may require one- or two-base size resolution by electrophoresis.

20 Rapid DNA sequencing methods currently in use rely on size fractionation of single-stranded DNA fragments on polyacrylamide gel. Both enzymatic sequencing techniques, in which random-termination fragments are generated enzymatically in the presence of dideoxynucleotides (Sanger), and
25 chemical methods in which random-termination fragments are generated chemically (Maxam), rely on discrimination of fragment size differences of one base. However, at larger fragment sizes, it becomes progressively more difficult to match and compare fragment peaks, and the precise base number of any large peak becomes more uncertain.
30

In the various high-resolution DNA fractionation methods described above, it is useful to include known-size reference fragments in the fractionated material, for cali-

brating fragment sizes on the gel. Typically, the size standards are generated by restriction digestion of plasmids with known restriction-fragment sizes, where the fragments are pre-labeled with a selected reporter, such as a
5 radioactive or a fluorescent reporter.

One limitation of this type of size standard is that both strands of the digest fragment are labeled and therefore detectable in the gel. Typically, the opposite labeled strands have slightly different migration rates on
10 the gel, giving a broadened or even double band at the reference fragment which makes accurate size determination more difficult. In addition, the sizes of the fragments which can be produced by this approach are limited to the relative positions of the restriction sequences in the
15 digested fragment.

4. Summary of the Invention

It is one general object of the invention to provide a size-calibration fragment mixture for use in high-resolution electrophoretic fractionation of polynucleotide fragments.
20

It is another object of the invention to provide a method of preparing and using such a fragment mixture.

The invention includes, in one aspect, a polynucleotide strand-pair mixture containing two or more polynucleotide strand pairs, where each pair has one strand having a selected number of polynucleotide bases and a detectable reporter in its 5'-end region, and a complementary strand which lacks this reporter. The different strand pairs in
25 the mixture have different selected sizes (numbers of basepairs).
30

For use in size calibration over a wide range of analyte fragment sizes, the number of bases in the repor-

ter-containing strands in the strand pairs preferably differ from one another by a multiple of a selected number of bases, typically 10-100 bases. Where the analyte fragments are fractionated by high-resolution polyacrylamide gel electrophoresis in the presence of a denaturant, such as urea, the fragments preferably have sizes less than about 1,200 bases.

In one preferred embodiment, for use in quantitating the amount of analyte fragments, the concentration of each strand pair fragment in the mixture is known, and one strand of each fragment is labeled with a fluorescence reporter. The concentration of analyte polynucleotides can be estimated, after electrophoretic separation, by comparing the total fluorescence emission signal of analyte peaks with those of adjacent-size calibration strands.

The invention also includes a method of producing a polynucleotide fragment mixture for use in size-calibrating polynucleotide analytes fractionated by electrophoresis. In practicing the method, a segment of template DNA having a selected number of basepairs is identified, and the segment-containing template is reacted with reporter-labeled and unlabeled single-stranded primers which are complementary to 3'-end regions of opposite strands of the segment, when the segment is in double-stranded form. The reaction is carried out under conditions which produce multiple rounds of primer-mediated duplex DNA replication, producing a duplex DNA fragment having the basepair composition of the segment, and one reporter-labeled strand only.

The steps of identifying a selected-size segment, and reacting the segment-containing DNA with reporter-labeled and unlabeled single-stranded primers are repeated with different, selected-size segments until a desired mixture of different-size fragments in the mixture is produced.

In one embodiment, each subsequently-identified segment contains the same basepair sequence as the previously identified segment, plus an end sequence of at least about 10 basepairs. In this embodiment, the reporter-labeled primer may be homologous to the common-sequence end of the sequences, and the unlabeled primer, homologous to the 5'-end sequence of the opposite strand of each successively larger sequence. The fragments may be produced simultaneously in a reaction mixture containing the common-sequence primer and each of the individual-sequence primers. Alternatively, the fragments may be produced individually and combined to produce the desired mixture. In the latter method, each different-size fragment can be added in the mixture to a known final concentration.

In another embodiment, an excess of one or more labeled strands is produced, relative to the corresponding unlabeled complementary strands, through the use of a limiting amount of the unlabeled primer, relative to the amount of the corresponding labeled primer, in primer-mediated replication of the selected size segments.

In another embodiment, the two primers required for primer-mediated replication of the selected size segments may be labeled with different, distinguishable reporter groups, such as different fluorescent reporters (Fung et al.), giving strand pairs in which the two complementary strands are labeled with the different, distinguishable reporters, permitting study of their relative electrophoretic mobilities.

Also disclosed are improvements in genomic mapping and sequencing methods employing the strand-pair mixture of the invention.

These and other objects and features of the invention will become more fully apparent when the following detailed

description of the invention is read in conjunction with the accompanying drawings.

Brief Description of the Drawings

5 Figure 1 illustrates how the method of the invention may be practiced to generate a series of strand pair fragments having a common downstream end delimited by a labeled forward primer, and upstream end regions whose lengths increase in increments of 50 basepairs, delimited
10 by reverse primers;

 Figure 2 shows the polynucleotide sequence of the sense (+) strand of M13mp18, where the sequence complementary to the 5'-end labeled primer is double underlined and the sequences of the reverse primers are underlined;

15 Figure 3 illustrates the primer-initiated reactions which occur in forming the strand-pair fragments of the invention;

 Figure 4 is an electropherogram of a strand-pair mixture having labeled strands which differ from one
20 another by 50 bases, fractionated on a polyacrylamide gel under denaturing conditions;

 Figure 5 shows a portion of a plot of migration time as a function of size for the fragment separation in Figure 4;

25 Figure 6 shows an enlarged portion of an electropherogram, illustrating how the concentration of analyte peaks (indicated by dashed lines) can be quantitated by reference to adjacent labeled strands of the strand-pair mixture (solid lines);

30 Figure 7 shows an electropherogram of digest subfragments produced by digesting phage lambda DNA with each of three combinations of restriction enzymes (upper panels),

with strand-pair fragments (lower panel), fractionated on a polyacrylamide gel under denaturing conditions;

Figures 8A-8D show electropherograms of ddATP, ddTTP, ddGTP and ddCTP sequencing fragment mixtures, respectively, fractionated on polyacrylamide gels under denaturing conditions, with size calibration fragments indicated by the arrows;

Figure 9 shows an electropherogram of double-stranded fluorescence-labeled restriction fragments and double-stranded strand-pair fragments labeled with a second fluorescence label, and fractionated by agarose gel electrophoresis under non-denaturing conditions; and

Figure 10 shows electropherograms of the same digest subfragments (upper panels) and strand pair fragments (lower panel) as were used in Figure 7, separated in this case on a 2% agarose gel, under non-denaturing conditions.

Detailed Description of the Invention

I. Strand-Pair Mixture

The strand pair mixture of the invention includes a plurality of selected-size polynucleotide strand pair fragments, such as fragments 20, 22, and 24, shown at the top in Figure 1. These fragments are also designated F_{50} , F_{100} , and F_{150} , respectively, indicating fragment lengths of 50, 100, and 150 basepairs, respectively. Fragment 30, which is representative, includes a polynucleotide strand 34 which has a detectable reporter 36 (indicated at R) in its 5'-end region, and an opposite, complementary polynucleotide strand 32 which lacks this reporter.

The reporter-labeled strand has a known, selected number of bases, and the opposite strand preferably has the same number of bases, i.e., the strand-pair fragment is base-paired along its entire length. It is known, however,

that Taq DNA polymerase may add one unpaired base (dA) at the 3' end of a DNA chain.

A. Size-Calibration Mixture

5 In one embodiment, the strand-pair mixture is designed for use in size calibration for DNA (or RNA) fractionation by electrophoresis, over a selected nucleic acid size range. The size range typically extends from about 50 bases up to as high as 1,200 bases for polyacrylamide elec-
10 trophoresis under denaturing conditions, and from about 50 bases up to as high as 2,000 or more for agarose electrophoresis of duplex nucleic acid fragments under non-denaturing conditions.

Preferably the strand-pair fragments in this embodi-
15 ment differ from one another in size by a selected multiple of N bases, where N is typically between about 10-100 bases, e.g., 50 bases. The strand-pair fragments shown at the top in Figure 1 have sizes between 50-300 basepairs, in increments of 50 basepairs.

20 Figures 1-3 illustrate how the size-calibration strand-pair mixture can be prepared in accordance with the method of the invention. Shown at the bottom in Figure 1 is a template 38 used in the method. For illustrative purposes, the template shown here is the sense (+) strand of
25 an M13mp18 plasmid. The strand serves as the template strand in the method. This strand has a region 40 of known sequence, which is shown in Figure 2, extending from a 5' end to a 3' end in the sense indicated by the arrows.

Region 40, in turn, is divided into a plurality of
30 partially overlapping segments, such as segments 42, 44, 46, which have a selected number of bases (or basepairs if both strands of a duplex template are considered). Each successively larger segment includes the bases in the pre-

vious segment plus an additional 50 bases in the upstream direction, i.e., toward the 5' end of region 40 in Figure 1. Thus, each segment includes a common 3' end sequence and a unique 5' end sequence.

5 A common-sequence primer P_0 is complementary to the 5' end region of each template strand sequence. Primer P_0 terminates, at its 5' end, in a detectable reporter 36, such as the ROX (6-carboxy rhodamine X) fluorescence reporter which has been described (Fung). A variety of
10 detectable fluorescent, colorimetric, or radioactive reporters may be employed as the detectable reporter in the primer. Alternatively, the reporter may be a ligand, such as biotin, which is capable of binding by specific, high-affinity binding to an antiligand, such as streptavidin.

15 The particular P_0 primer for the M13 sense sequence shown in Figure 2 has the sequence: d(5'-ROX-TGT AAA ACG ACG GCC AGT-3'), where ROX is coupled via a linker to the 5'-phosphate on the terminal thymidine as shown.

 A second, reverse primer has the sequence of the 5'-
20 end region of each template-strand segment, i.e., in the template illustrated in Figures 1 and 2, the first segment 42 is bounded by P_0 and a second primer P_{50} , which has the sequence shown by underlining for the P_{50} primer. The specific P_{50} primer shown in the figure has the form: d(5'-
25 CTA GAG TCG ACC TGC AGG CAT GCA AGC TTG-3'). The two primers P_0 and P_{50} are used in producing fragment F_{50} , a 50-basepair fragment, according to procedures described below.

 Segment 44 in the template is a 100-base segment extending upstream from the 3'-end of template region 40 in
30 Figure 1. This segment is bounded by the two primers P_0 and P_{100} , the latter of which has the sequence shown by underlining in Figure 2. As above, P_{100} is unlabeled, and the two primers P_0 and P_{100} are complementary to the 3'-end

regions of opposite strands of segment 22, when the segment is in duplex form. The two primers are used in producing strand-pair fragment F_{100} , a 100-basepair fragment, as detailed below.

5 Similarly, each larger segment in the template, such as segment 46, is bounded by the primer P_0 and an upstream primer, such as primer P_{150} , whose sequence bounds the upstream end of that segment. The sequences of these additional primers P_{150} - P_{1000} are underlined in Figure 2. As
10 shown in Figure 1, each of these additional primers is used in conjunction with the common, reporter-labeled primer P_0 to produce the strand-pair fragments F_{150} to F_{1000} .

Figure 3 illustrates the reaction steps used in generating selected-size strand-pair fragments, in accordance
15 with the method of the invention. Shown at A in this figure is a portion of the single-stranded M13mp18 template 38, containing a selected-size segment, such as segment 48 (a 200 basepair segment), from the template. The 3' and 5' ends of the size-selected segment are shown, along with the
20 P_0 primer, which is complementary to the 3'-end of the selected segment. The template and selected pair of primers are employed in a fragment amplification method based on the PCR (polymerase chain reaction) amplification method disclosed in U.S. Patent No. 4,683,202. Details of
25 an exemplary reaction method are given in Example 1. The general reaction steps involve first mixing the template with the reporter-labeled primer and unlabeled primer which bound the selected segment, heating the DNA components to denature the template strands (necessary in the case of a
30 duplex template), and cooling the components under conditions which favor primer annealing to the template strand(s), as shown at A in Figure 3.

Additional reaction components, which may be added before or after primer annealing to the template strands, include all four deoxynucleotide, 5'-triphosphates (dNTPs) and a thermal-stable DNA polymerase, such as the polymerase
5 obtained from Thermus aquaticus. The polymerase operates, in the presence of the dNTPs, to extend the primer in the 5' to 3' direction by the addition of bases complementary to the template strand, forming duplex fragments which include the selected template fragment plus a strand
10 extending in a 5'-3' direction from the P_0 sequence as shown at B in the figure. As seen, the newly formed strand 54 is reporter labeled.

Heating to denature the newly formed duplex frees the labeled product strand 54, which can then anneal with the
15 unlabeled primer P_{200} , as shown at C in Figure 3. Polymerization gives the new duplex shown at D, with an unlabeled strand 56 corresponding to the size-selected segment 48 of template 38. Denaturation of this duplex and annealing the unlabeled strand with labeled primer P_0 , as shown at E in
20 Figure 3, then permits polymerization to give the duplex shown at F, consisting of a newly synthesized, labeled product strand 58 and the unlabeled template strand 56 corresponding to the selected segment 48 of template 38, both strands having the selected number of basepairs.

25 The above denaturing, annealing, and polymerizing steps are repeated, with each of the six strands shown at B, D and F being annealed with one of the two primers in the reaction mixture. It will be appreciated that the reporter-labeled strand at B will be annealed at its 3' end
30 with the unlabeled primer, forming, on polymerization, the duplex shown at D, while the unlabeled strand at B will anneal to the labeled primer to repeat the process shown at A and B. Similarly, the primer-induced, unlabeled strand

at D will be annealed at its 3' end with the reporter-labeled primer, again forming on polymerization the duplex shown at F, while the labeled strand at D will be annealed at its 3' end by the unlabeled primer, again forming on polymerization the duplex shown at D. The duplex shown at F contains labeled and unlabeled strands of equal length; on denaturation, the unlabeled strand will anneal to the labeled primer (as at E), to give on polymerization the same duplex shown at F; the labeled strand will anneal to the unlabeled primer, also giving, after polymerization, another duplex identical to that shown at F.

The denaturing, annealing, and polymerization cycle is repeated N times, forming approximately 2^N strand-pair fragments of the type just described having the basepair sequence of the selected template segment, and a single reporter-labeled strand. After terminating the reaction, the selected-size fragments can be freed of primer and other unwanted reaction components by centrifugal filtration using commercial membrane devices such as Centricon™ (Amicon) or Centri/Por (Spectrum Medical Industries), washing with Tris-EDTA buffer (pH 8). The purified strand-pair components are precipitated with cold ethanol and resuspended in a suitable buffer to a desired DNA concentration.

Each selected-size strand-pair fragment, such as the F_{200} fragment, is formed by the above method, using the template and appropriate set of primers, such as the labeled P_0 primer and unlabeled P_{100} primer described above. The final strand-pair mixture is formed by combining the individual strand-pair fragments. Thus, for example, a strand-pair mixture for size calibration in the 50-600 basepair range would be prepared by making each of the individual fragments F_{50} , F_{100} , ..., F_{600} , and combining these into a single mixture.

In practice, F_{50} is more conveniently prepared by automated synthesis of the two strands, e.g. using an Applied Biosystems Model 380B DNA synthesizer. The strand to be labeled with the reporter is synthesized with a linker group at its 5' end, to which the reporter group is subsequently attached. After purification by conventional methods, the two strands are combined in a one-to-one ratio to give a duplex identical to that produced by primer-mediated synthesis using a size-selected template. Automated synthesis of F_{100} is also practical. These synthetic fragments are resuspended at the desired concentration and combined with the other fragments made as described above.

A size-calibration standard was prepared as described above, with strand-pair fragments having selected size between 50-1,000 basepairs, at 50 basepair intervals. The fragments were denatured and fractionated by polyacrylamide gel under denaturing conditions, as detailed in Example 2. Fluorescence emission of the bands was measured at a position about 25 cm from the initial sample position, with the results shown in Figure 4. As seen, all of the reporter-labeled strands except two migrated as single bands with well-defined peak positions. The two exceptions -- at 350 and 400 base sizes -- gave double peaks evidencing fragments with one basepair size differences. The single peaks allows the migration time of any given size fragment to be unambiguously determined. When the peak migration times in Figure 4 are plotted against peak size, the plot shown in Figure 5 is obtained. The non-linear behavior is due to variation in migration rate as a function of strand size.

To determine the size of any unknown analyte fragment which is co-electrophoresed with the size-calibration fragments, the migration time of the analyte peak is measured and this peak time is plotted on the Figure 5 time/size

plot. Thus, for example, an analyte peak with measured migration time of 364.5 minutes is determined to have a 288 base size. It will be appreciated that the frequent and regularly spaced calibration peaks, in conjunction with the sharp peaks achieved with a single-labeled strand, allows unambiguous determination of single-strand analyte peaks based on their migration rates in a denaturing gel. Applications of the strand-pair mixture of the invention for fragment size determinations are described in Section II below.

As an alternative method for producing the above described size-calibration mixture, a single template, such as template 38, is mixed initially with all of the primers used in generating the desired selected-size fragments. For example, in the case above, the M13 template is mixed with the P_0 , P_{50} , P_{100} , ... P_{1000} primers in the presence of the dNTPS and thermal-stable polymerase, and the mixture taken through several cycles of denaturation, annealing, and polymerization. All of the strand-pair fragments are thus formed simultaneously in one primer-initiated amplification reaction. The method has the advantage of simplicity; however, the relative concentrations of the different-size fragments formed in the reaction may be difficult to control, and this may preclude the use of fragment mixture for quantitating electrophoresis peaks, as described below.

It will also be appreciated that the different-size strand-pair fragments in the mixture of the invention may be formed using a different template for each fragment. This method would require an independent pair of a labeled and unlabeled primers for each different template sequence.

B. Size Calibration Mixture for DNA Quantitation

In one preferred embodiment of the invention, for use in quantitating the amounts of individual analyte peaks on electrophoresis, each strand-pair fragment in the mixture is present at a known concentration. Typically in such a mixture, all of the strand-pair fragments are present at the same concentration. The mixture is prepared by combining known amounts of each of the individual strand-pair fragments prepared as above. In one preferred embodiment, each of the strand-pair fragments in the mixture has the same concentration, in a concentration range between about 1 and 10 $\mu\text{g/ml}$, corresponding to about 10 pmol/mL.

The use of the above strand-pair mixture for quantitating the amount of an unknown analyte by gel electrophoretic fractionation is illustrated in Figure 6, which shows an idealized portion of an electropherogram containing size calibration peaks for 250 and 300 bases, and an intermediate analyte peak, indicated at 70. The amount of each of the two size-calibration peaks applied to the gel is known from the known concentration of each fragment in the strand-pair mixture.

The total fluorescence emission associated with each band may be more accurately determined by sampling fluorescence emission across the length and width of the band, and using the sampled values to construct a three-dimensional fluorescence emission surface. Total fluorescence is then calculated from the volume defined by the emission surface, and the calculated volume is plotted as a function of electrophoretic migration rate. A variation in measured fluorescence emission with migration time will be observed, due to the change in peak shape as a function of fragment size, as illustrated in Figure 4, and this variation can be stan-

standardized from the known amount of material in each band. It will be appreciated that the frequent, regularly spaced and sharp strand peaks provided by the strand-pair mixture of the invention, combined with the known DNA amount associated with each peak, allows the relationship between peak fluorescence emission, DNA amount and migration distance on a denaturing gel to be accurately calibrated.

From the fluorescence emission plot just described, the amount of DNA in any analyte peak can be determined from the measured fluorescence emission surface and migration rate of that peak. If the fluorescence reporter attached to the analyte strand is different from that in the size-calibration strands, an additional correction for fluorescence efficiencies will also be required. The correction factor can be determined readily from sample peaks containing known amounts of each reporter.

C. Small-Increment Fragment Mixture

In another embodiment, the strand-pair mixture of the invention includes two or more strand-pair fragments having defined sizes which differ from one another by a small number of bases, typically 1-10 bases. The mixtures are designed for use, for example, in detecting small base-number mutations in a genomic fragments having defined-sequence ends, or in detecting polymorphisms in genomic sequences containing variable numbers of tandem repeat sequences (VNTRs). In each of these applications, the strand-pair fragments are tailored in size to provide strand sizes which span the size of the analyte(s) of interest.

For example, a strand-pair mixture for use in detecting a one- or two-base mutation in a genomic segment would contain strand pairs with sizes corresponding to the normal

(non-mutated) genomic segment \pm one or two bases, respectively. As another example, a strand pair mixture for use in detecting polymorphisms of variable numbers of tandem repeats (VNTRs) would contain size markers corresponding to upper and lower expected sizes of genomic segments having minimum and maximum expected numbers of VNTRs, respectively.

Such strand-pair mixtures are prepared as described above, preferably using a single template with substantially overlapping segments which differ in base number from one another by the selected number(s) of bases, e.g., 1-30 bases.

II. Electrophoretic Methods Involving Single-Strand DNA

The strand-pair mixture described above is designed for use in a variety of electrophoretic methods in which DNA analyte fragments are fractionated in single-stranded form by electrophoresis. The mixture of the invention allows analyte sizes to be determined exactly (to a one-base accuracy), and optionally allows the amount of DNA in the bands to be quantitated.

The electrophoretic methods are carried out in any electrophoretic system capable of resolving analyte fragments to a resolution of one base, i.e., of separating fragments which differ from one another in size by a single base. One preferred system is a polyacrylamide slab gel in a denaturant buffer, as detailed below. Single base resolution has also been achieved by capillary electrophoresis using either a polyacrylamide matrix, or a solution of a hydroxylated polymer as the separation medium, as described in co-owned U.S. Patent application for "Nucleic Acid Fractionation by Counter-Migration Capillary Electrophoresis," Ser. No. 390,631 filed August 7, 1989.

A. Genomic Mapping

As a general strategy for gene mapping a large genomic region, such as an entire chromosome, the region is first fragmented, and the fragments are cloned to form a genomic library. The library clones are then examined to identify regions of sequence overlap between cloned inserts, with the aim of identifying and ordering clones with overlapping sequences. Once several clones with consecutively overlapping regions are identified, the basepair sequences of the clones can be determined, and the sequences can then be matched to produce extended basepair sequences.

One method which has been used for identifying regions of sequence overlap in cloned inserts uses restriction analysis to characterize the restriction fragment sizes in a digested insert, then matches the digest fragment sizes among insert fragments to identify the fragments with possible sequence overlap. Carrano et al have described such a method in which each cloned insert is digested with different pairs of restriction enzymes, generating two or more sets of restriction fragments for each insert. Each fragment set is labeled with a distinctive fluorescence reporter, and the fragment sets are run together in a common electrophoresis lane, as described in Example 3, in a polyacrylamide gel under denaturing conditions.

The resulting peaks are monitored by fluorescence emission and signal processing techniques which allow each set of fragments to be individually monitored. A typical electropherogram generated by this method is shown in Figure 7, where the three upper lines represent electropherograms of HindIII/HaeIII, BamHI/HaeIII, and EcoRI/HaeIII fragments of phage lambda DNA as indicated, and the lower trace represents size-calibration fragment peaks.

The success of the fragment matching method for genomic mapping depends on the ability to accurately determine the sizes of the digest fragments by electrophoretic fractionation. In particular, it is important to determine the exact size (number of bases) of each fragment, for accurate size matching among digest fragments from different clonal inserts.

In the present invention, the ability to determine fragment size to an accuracy of one base is accomplished by co-electrophoresing the digest fragments with the size-calibration strand-pair mixture described in Section I, where the reporter in the labeled size-calibration strands is preferably distinguishable from the reporter(s) used for labeling the digest fragments. In the Figure 7 electropherogram, the size-calibration strands and each of the three restriction digests are labeled with a separate fluorescence reporter, which allows the four samples to be separately detected in a single electropherogram.

The sizes of the digest fragments in the electropherogram are determined, as described above, by plotting the known-size strands as a function of migration time, and using this plot to determine analyte fragment size.

B. DNA Sequencing

Accurate size determination of single-stranded DNA is also required in DNA sequencing methods. In a standard dideoxy sequencing method, chain elongation in the presence of a selected dideoxynucleoside triphosphate (ddNTP) is used to generate random-size fragments which terminate at the selected ddNTP, where the primer employed in the elongation reaction is fluorescence labeled, to label the fragments. Each of the four ddNTP reaction mixtures is fractionated by electrophoresis and the fragment lengths

are determined to construct the unknown sequence (Sanger). The four reaction mixtures may be co-fractionated in a single lane, where each of the four reaction mixtures are labeled with a distinguishable fluorescence probe (Smith).

5 Alternatively, the four reaction mixtures may be fractionated in parallel, in side-by-side lanes.

Figures 8A-8D illustrate fragment electropherograms for each of the four ddNTPs in a typical dideoxy sequencing method. The sequence is "read" conventionally by identifying and assigning a fragment size to each peak in the four reactions mixtures, and counting the peak fragment sizes in numerical succession. One limitation of this method heretofore has been the uncertainty in fragment size, particularly as the fragment size extends beyond about 400 bases, leading to loss or misidentification of bases in the sequence.

In the present method, the fragments produced by random termination in the above sequencing method are fractionated by gel electrophoresis in the presence of the size-calibration, strand-pair mixture of the invention. In one embodiment, illustrated in Figures 8A-8D, the four ddNTP reaction mixtures are separately fractionated, each in the presence of the strand-pair mixture. In this embodiment, the random-size termination fragments and the strand-pair fragments are labeled with distinguishable fluorescence reporters, as described in Example 4. In Figures 8A-8D, the traces represent the measured fluorescence emission from the random-termination strands and the reporter-labeled size-calibration strands (arrows).

30 For each of the four electropherograms, the size-calibration peaks are plotted as a function of strand size, and this plot is then used to determine fragment size for each of the random-termination strands in that electropherogram.

As described above, this method provides precise size determination up to 700 bases or more, allowing accurate sequence determination for fragments up to 700 bases or greater in size.

5

III. Applications to Double-Strand DNA Electrophoresis

In another general method, the strand-pair mixture of the invention is employed as a size-calibration standard for double-stranded DNA analytes fractionated by electrophoresis. The electrophoresis is typically carried out by agarose gel electrophoresis in a slab gel, although separation may be carried out in other electrophoretic systems capable of resolving double-stranded DNA fragments with high resolution, generally within one or two basepairs.

One application of the duplex-fractionation method is for detecting relatively small size changes in restriction-enzyme digest fragment, where a duplex fragment is digested partially or completely with a selected restriction enzyme or set of enzymes, and the resulting digest fragments are characterized on the basis of size. This application may involve, for example, detecting small bases number mutations in a restriction fragment, or small size changes related to restriction fragment length polymorphisms (RFLPs), or polymorphisms involving variable numbers of tandem repeats (VNTRs), as discussed above.

Another application is in genomic mapping, where a number of genomic inserts are characterized on the basis of restriction-fragment sizes, to map regions of overlap among the inserts. This approach is similar to the one discussed in Section II, except that the digest fragments are fractionated in double-stranded form. Figure 10 shows a portion of an electropherogram of the same phage lambda restriction digest fragments as in Figure 7, here fractio-

nated in double-stranded form on a 2% agarose horizontal slab gel. The fragments were digested and labeled with a fluorescence reporter, and mixed with a double-strand size calibration mixture prepared as in Section II and labeled with a second fluorescence reporter, following the procedures detailed in Example 5. The size-calibration fragments with sizes between 100 and 1000 basepairs are indicated in the figure.

To determine the sizes of the analyte fragments, the peak positions of the size-calibration fragments are plotted as a function of fragment size, and the analyte sizes are determined from their peak migration rates, using this plot. As in the analogous application involving single-stranded fragments, the size-calibration mixture of the invention increases the accuracy of fragment size determination by providing a series of uniformly spaced size markers whose frequent spacing throughout a size region of interest allows an accurate plot of peak migration time as a function of fragment size. It is recognized that some accuracy in size determination will be sacrificed in fragment separation by agarose electrophoresis under non-denaturing conditions, due to peak broadening, seen, for example, by comparing peak widths in Figure 10 with those in Figure 7. However, duplex-fractionation method is generally applicable to fragment separation of larger fragments, e.g., above about 700-1,000 bases, which may not be well resolved by polyacrylamide gel under denaturing conditions.

From the foregoing, it can be seen how the various objects and features of the present invention are met. The strand-pair mixture provides, in one aspect, a series of uniformly spaced size markers which can be used for constructing a plot of migration distance vs fragment size, allowing any analyte to be sized accurately with reference

to two closely spaced standard markers. Moreover, the spacing between the standard markers can be easily selected for greater accuracy, if needed. That is, size-dependent migration effects can be refined with increasing numbers of strand-pair sizes.

The strand-pair fragments of the invention contain a single reporter-labeled strand, and thus give sharp, single-peak bands when used in a denaturing electrophoresis system. In one preferred embodiment, the individual-size fragments are present in known amounts, allowing for construction of a plot of peak reporter signal as a function of migration distance. From this plot, quantitative determination of analyte fragments, based on total reporter signal and migration distance, can be made.

The strand-pair fragment mixture can be conveniently prepared, in accordance with the method of the invention, from a single template, a single reporter labeled primer, and a group of second (reverse) primers whose sequences are selected to produce desired fragment sizes.

The following examples illustrate methods for preparing and using the strand pair fragment mixture of the present invention. The examples are intended to illustrate, but in no way to limit the invention.

25

Example 1

Preparing a Size Ladder Strand-Pair Mixture

A. Preparation of Single-Strand Primers

Size-fragment primers were prepared by standard automated DNA synthesis methods, using an Applied Biosystems Model 380B DNA synthesizer. The unlabeled primers were purified using OPC™ cartridges (Applied Biosystems), fol-

25

lowed by drying and resuspension at the desired concentration ($20\mu\text{M}$).

The P_0 primer was synthesized with a linker group (Aminolink 2™) coupled to the 5' T residue as the final step of automated synthesis. After deprotection and evaporation of the ammonia solution, the ROX fluorescent reporter was added by reaction of the linker-modified primer with ROX-NHS ester (Applied Biosystems) in sodium carbonate/sodium bicarbonate buffer (250 mM, pH 9) overnight at room temperature. Excess fluorescent dye was removed by Sephadex™ G-25 (Pharmacia) gel filtration, and preparative high-performance liquid chromatography (prep HPLC) was used to remove other impurities, including any primer that failed to couple with the fluorescent reporter. Prep HPLC was carried out on an Applied Biosystems 150A DNA separation system, using an Aquapore RP-300 column (10 × 250 mm), eluting with 0.1 M triethylammonium acetate and a gradient of 5 to 35% acetonitrile over 30 minutes. The flow rate was 4 ml/min. The desired fraction was collected, and its purity confirmed by HPLC. The purified, dye-labeled primer was concentrated and resuspended at $10\mu\text{M}$ final concentration.

The labeled and unlabeled strands of F_{50} and F_{100} were prepared analogously, as explained above. The P_0 and P_{50} -
25 P_{1000} primer sequences are shown in Table 1 below.

SUBSTITUTE SHEET

Table 1

	Rev Primers	Sequence (5' - 3')	ODU/ μ mol
5	P ₀	ROX-TGT AAA ACG ACG GCC AGT	3
	P ₅₀	CTA GAG TCG ACC TGC AGG CAT GCA AGC TTG	3
	P ₁₀₀	AAA CAG CTA GAG TCG ACC TCG AGG CAT GCA AGC TTG	4
	P ₁₅₀	CCG GCT CGT ATG TTG TGT GGA ATT GTG AGC	3
	P ₂₀₀	ATG TGA GTT AGC TCA CTC ATT AGG CAC CCC	3
10	P ₂₅₀	GCA CGA CAG GTT TCC CGA CTG GAA AGC GGG	3
	P ₃₀₀	ATA CGC AAA CCG CCT CTC CCC GCG CGT TGG	10
	P ₃₅₀	TCA GCT GTT GCC CGT CTC ACT GGT GAA AAG	3
	P ₄₀₀	AGC GTG GAC CGC TTG CTG CAA CTC TCT CAG	3
	P ₄₅₀	CCG ATT TCG GAA CCA CCA TCA AAC AGG ATT	3
15	P ₅₀₀	CAA CAC TCA ACC CTA TCT CGG GCT ATT CTT	12
	P ₅₅₀	GAC GTT GGA GTC CAC GTT CTT TAA TAG TCG	3
	P ₆₀₀	GAT GGT TCA CGR AGT GGG CCA TCG CCC TGA	3
	P ₆₅₀	TCC GAT TTA GTG CTT TAC GGC ACC TCG ACC	11
	P ₇₀₀	GTT CGC CGG CTT TCC CCG TCA AGC TCT AAA	10
20	P ₇₅₀	AGC GCC CTA CGG CCC GCT CCT TTC GCT TTC	25
	P ₈₀₀	TAA GCG CGG CGG GTG TGG TGG TTA CGC GCA	3
	P ₈₅₀	TAT ACG TGC TCG TCA AAG CAA CCA TAG TAC	3
	P ₉₀₀	TAA TCG GCC TCC TGT TTA GCT CCC GCT CGT	25
	P ₉₅₀	TAA AAA CAC TTC TCA AGA TTC TGG CGT ACC	3
25	P ₁₀₀₀	GTT AAT TTG CGT GAT GGA CAG ACT CTT TTA	3

B. Preparation of Strand-Pair Mixture

30 M13mp18 (+) single-stranded DNA (1 mg/mL) was obtained from Promega Corporation (Madison, WI). 10× PCR amplification buffer (Perkin Elmer Cetus) contained 100 mM Tris-HCl pH 8.3, 500 mM KCl, and 0.01% gelatin. A dNTP mix was prepared by combining dATP, dCTP, dGTP, and dTTP at equal
35 concentrations (1.25 mM each).

In a 0.5 mL microcentrifuge were combined water (139 μ L), 10× amplification buffer (20 μ L), 25 mM magnesium chloride solution (16 μ L, 2 mM final reaction concentration), forward primer P₀ (5 μ L, 50 pmol), reverse primer P₃₀₀
40 (2 μ L, 40 pmol), dNTP mix (16 μ L, to give 100 μ M of each dNTP in the reaction), M13mp18 solution (1 μ L, 1 μ g), and 5 units (1 μ L) of Thermus aquaticus DNA polymerase (Tag

polymerase, Perkin Elmer Cetus). The mixture was covered with two drops of mineral oil, and the closed tube placed in a preheated thermocycler (Perkin Elmer Cetus DNA Thermal Cycler) at 95°C for 5 minutes (DNA duplex denaturation).
5 The mixture was then subjected to cycles of heating to 95°C for 1 minute, cooling to 60°C for 30 seconds (primer annealing), and heating to 72°C for 1 minute (primer extension by Taq polymerase). The replication reaction, each cycle involving successive denaturation, primer
10 annealing, and DNA polymerization steps, was repeated a total of 35 times.

The 300-base duplex fragments produced in the reaction were freed of single stranded primers by dilution into 1.0 mL water and filtration on a Centricon-100 microconcentra-
15 tor (Amicon), washing 3 times with 2.0 mL of water according to the manufacturer's instructions. The purified DNA was recovered by inverting the microconcentrator and collecting the product in the concentrator cap. The product (recovered in about 60 µL water) was assayed by
20 capillary electrophoresis (Applied Biosystems 270A with SeptraGene™ 500 buffer, monitored by absorption at 260 nm).

The primer-initiated amplification described above was repeated individually with each of the primers P_{100} - P_{1000} . The final reaction components for each primer were freed of
25 primer material, and concentrated as described above. Each fragment solution was analyzed for purity and relative concentration by capillary electrophoresis as described above. A fragment mixture was prepared by combining aliquots of each fragment solution in proportion to their
30 relative concentrations, to a final concentration of approximately 1-10 µg/mL for each size fragment. Each fragment in the mixture was characterized by (a) a known number of bases between 50-1000, in increments of 50 bases,

and (b) one strand which has a 5' end ROX fluorescence reporter, and an opposite, unlabeled strand.

Example 2

5 Fractionating a Size Ladder Mixture by Polyacrylamide Electrophoresis

A slab polyacrylamide gel containing 6% polyacrylamide was prepared by standard methods (User Bulletin Number 16 for the Model 373A DNA Sequencing System, Applied Biosystems, Inc., Foster City, CA, January 1991) between 5 mm high-
10 optical quality glass plates, to slab dimensions of 0.4 mm thick, 22 cm width, and 40 cm length. The gel was prepared in a standard TBE buffer (0.089 M Tris base, 0.089 M boric acid, 0.002 M EDTA) containing 8 M urea. An aliquot of the
15 strand-pair mixture prepared in Example 1 was dried down and resuspended in 5 μ L of loading buffer (containing 5 parts v/v deionized formamide to one part 50 mM EDTA solution, pH 8.0); the mixture was heated for 2 minutes at 90°C to denature the strand-pair fragments, and 5 μ l of the
20 denatured strand-pair mixture was added to a sample well at the top of the gel.

Electrophoresis was carried out on a Model 373A DNA Sequencing System (Applied Biosystems, Inc, Foster City, CA), using a standard Tris-borate buffer as the running
25 buffer. Electrophoretic voltage was 1300-1400 V, at approximately 20 ma with the power regulated at 30 W and the temperature regulated at 40°C. The peaks were monitored by a scanning laser fluorometer positioned to detect fragments exactly 25 cm from the sample origin in the gel.
30 Details of the fluorometer and fluorescence-emission signal processing system have been published (Connell). The electropherogram generated from the sample is shown in Figure 4. The double peaks at 350- and 400-base fragment

SUBSTITUTE SHEET

sizes are presumably due to the action of the Tag polymerase, which may add one additional base (dA) beyond the end of the template strand.

5

Example 3Fingerprinting for Genomic Mapping

Lambda DNA was digested with either Hind III, Bam HI, or Eco RI restriction endonuclease, and the restriction fragments were 5'-end labeled by ligation to labeled oligodeoxynucleotides according to published methods (Carrano). Each restriction/ligation reaction contained lambda DNA (6.4 μ g, 0.2 pmol), the labeling oligodeoxynucleotide and its complement or "splint" (30-40 pmol of each, for a 10-fold excess relative to the number of restriction cut ends expected), restriction enzyme (6 units per μ g of DNA), T4 DNA ligase (1 unit per μ g of DNA), and ATP at 1 mM, in a total volume of 40 μ L, buffered with 1x restriction digest buffer supplied by the enzyme manufacturer (Promega Corporation, Madison, WI). The 18-mer labeling oligodeoxynucleotides had the same sequence as the primer P₀ shown in Example 1, above, except that the fluorescent label used with Hind III was FAM, the label used with Bam HI was JOE, and that used with Eco RI was TAMRA (Connell). The three complementary "splint" oligodeoxynucleotides were 22-mers, with four extra bases at the 5'-end chosen to complement the 5'-overhang created by the corresponding restriction enzyme:

30

Hind III: 5'-A GCT ACT GGC CGT CGT TTT ACA-3'

Bam HI: 5'-G ATC ACT GGC CGT CGT TTT ACA-3'

35

Eco RI: 5'-A ATT ACT GGC CGT CGT TTT ACA-3'

SUBSTITUTE SHEET

Each reaction was incubated overnight at 37°C. The labeled fragments were then further digested by incubation for an additional two hours at 37°C with restriction endonuclease Hae III (6 units per μg of DNA). The reactions were then
5 stopped by addition of 0.8 μL of 500 mM EDTA solution (pH 8.0).

Aliquots of the three fragment mixtures were combined in a DNA concentration ratio of 1:1:2 for the FAM-, JOE- and TAMRA-labeled fragments. The ROX-labeled strand-pair
10 fragments from Example 1 were added to this mixture in an approximately 1:1 DNA concentration ratio. The pooled sample was precipitated with 0.1 volume of 3 M sodium acetate (pH 5.5) plus 2.5 volumes of absolute ethanol. After centrifugation at 12,000 rpm for 20 minutes at 4°C,
15 the supernatant was decanted. The precipitated sample was washed by addition of 250 μL of cold 70% ethanol followed by centrifugation for 5 minutes. The supernatant was again decanted, and the residue dried briefly in a vacuum evaporator. It was resuspended in gel loading buffer (see
20 Example 2) and denatured by heating for 2 minutes at 90°C. The resuspended sample was applied to a lane of an 8 M urea polyacrylamide slab gel prepared as in Example 2. Electrophoresis and fluorescence-peak monitoring were carried out as in Example 2, with the fluorescence emission from each
25 of the four different fluorophores being monitored by four different band-pass filters as described by Connell et al. Data acquisition was carried out using DNA sequencing software provided with the Model 373A Sequencer (Applied Biosystems, Inc., Foster City, CA). Data analysis to
30 resolve the peaks associated with each of the fluorophores was carried out using the Model 672 GENE SCANNER™ Analysis Software for the DNA Sequencer (Applied Biosystems, Inc., Foster City, CA).

SUBSTITUTE SHEET

For comparison, samples of the restriction digests described above were analyzed on the same polyacrylamide gel, using a ROX-labeled size standard prepared by restriction endonuclease PstI digestion of phage lambda DNA (GENESCAN-2500 ROX, Applied Biosystems, Inc., Foster City, CA).

The electropherogram obtained is shown in Figure 7, where the FAM (HindIII/HaeIII, JOE (BamHI/HaeIII), TAMRA (EcoRI/HaeIII), and ROX (size fragments) fluorescent signals are as indicated. The positions of the ROX-labeled size-calibration strands was plotted as a function of strand size, and this plot was used to establish the basepair numbers of each of the FAM-, TAMRA, and JOE-labeled fragment strands. The observed and expected basepair number of each of the latter fragments is given in Table 2 below. For almost every expected restriction digest band, the DNA standard (Selected Size Ladder) prepared according to the method of the present invention gives more accurate fragment sizing than does the conventional method (GENESCAN-2500). The difference in accuracy is summarized by the chi squared values, which measure the relative discrepancies between observed and expected values (the larger the chi squared value, the greater the deviations from the expected values). Note that the 697 basepair Hind III/Hae III fragment migrates anomalously; while its actual size cannot be determined correctly with either standard, its characteristic mobility can nonetheless be used to confirm its identity.

32

Table 2 Fragment Size			
	Selected Size Ladder Found	Hind III/Hae II Expected	GENESCAN-2500 Found
5	133.1	125	140.7
	144.9	136	150.8
	223.0	229	226.5
	369.6	371	356.7
	613.1	615	608.1
10	577.3	697	570.7
	875.7	872	875.8
	942.1	948	946.7
	12.11	chi squared	19.01
	Selected Size Ladder Found	Bam HI/Hae III Expected	GENESCAN-2500 Found
15	99.7	99	103.3
	128.3	124	133.6
	206.5	203	211.0
	286.0	288	284.1
20	336.9	339	334.3
	439.9	439	433.3
	457.4	453	450.0
	819.1	816	819.7
	0.54	chi squared	3.11
	Selected Size Ladder Found	Eco RI/Hae III Expected	GENESCAN-2500 Found
25	44.9	43	46.3
	127.2	119	127.2
	151.4	147	157.3
	257.2	256	255.5
30	307.2	308	306.2
	541.7	537	533.8
	729.4	733	733.0
	1040.6	1038	1044.3
35	1.14	chi squared	4.87

SUBSTITUTE SHEET

Example 4DNA Sequencing

The M13mp18(+) plasmid, in single-stranded form, was used as the template for DNA polymerization, in combination
5 with the P₀ primer noted above. Chain elongation and random-base termination in the presence of a selected-base 2',3'-dideoxynucleoside 5'-triphosphate was carried out according to standard procedures (Sanger).

Briefly, the primer used for each reaction mixture was
10 a FAM-labeled primer. The template and primer in each of the four reaction mixtures were heated to 60°C for 10 minutes, then cooled slowly to room temperature. The chain elongation reactions were each carried out in the presence of all four deoxynucleoside 5'-triphosphates (dNTPs), Taq
15 DNA polymerase, (Promega Corporation, Madison, WI) and the selected dideoxynucleoside-triphosphate (ddNTP) for 30 minutes at 60°C. The reaction volumes were precipitated by addition of sodium acetate (to 0.3 M) and 2.5 volumes of ethanol, followed by centrifugation at 12,000 rpm for 20
20 minutes at 4°C. The supernatant was decanted, and 250 µL of cold 70% ethanol was added to each tube. The tubes were centrifuged an additional 5 minutes, the supernatant again decanted, and the products dried briefly.

Each of the four dideoxy termination reaction mixtures
25 from above was combined with 1 µL (approximately 5 fmol) of the ROX-labeled strand pair mixture from Example 1. Each of these mixtures was then denatured by addition of 5 volumes of deionized formamide, with heating for 2 minutes 90°C.

30 A polyacrylamide slab gel in Tris-borate buffer, and containing 8M urea was prepared as in Example 2. To the top of the gel, at four different wells, were added 5 µl of the denatured DNA mixtures. Electrophoresis was carried out under the conditions described in Example 2. The fluo-
35 rescent-labeled DNA peaks were monitored, for each lane, at

fluorescence emission wavelengths effective to resolve fluorescence emission from the FAM and ROX reporters. Data collection was performed on a Macintosh® IICx computer (Apple Computer, Cupertino, CA) using the instrument's DNA
5 sequencing software. The results, plotted for each lane, are shown in Figures 8A-8D, for ddATP, ddTTP, ddGTP, and ddCTP terminations, respectively.

For each lane, the migration times (to detection) of the ROX peaks were plotted as a function of fragment size.
10 The number of bases in each of the FAM peaks was then determined from this plot, using the Model 680A Contig Mapper software (Applied Biosystems, Foster City, CA) running on a VAX computer (Sun Microsystems, Mountain View, CA). From the results, the fragment sequence was deter-
15 mined. The sequence could be read with reasonable accuracy (97.5%) for 600 bases from the starting point twelve bases downstream of the primer binding site. In 8 instances, the correct base could not be read from the data, and in 7 instances the correct order of two bases could not be
20 determined (in one of the cases, the data appeared to require an extra base, or insertion error). These results compare favorably to manual or automated sequencing, as carried out with no internal-lane size standard.

25

Example 5

Restriction Digest Analysis on Agarose Gels

Purified pBR322, obtained from Promega Corporation (Madison, WI), was digested to completion with AluI. The 18-base, single-stranded oligonucleotide from Example 3
30 (labeled at its 5' end with a FAM fluorescence reporter) was annealed with a complementary oligonucleotide. The Alu I fragments were ligated with FAM-labeled duplex fragments, as in Example 3, to form end-labeled pBR322 digest fragments (GENESCAN-1000™ FAM). The fragments were diluted in

35

10 mM EDTA solution (pH 8.0) to a working concentration of 1 nM.

A calibration mixture containing ROX-labeled strand pair fragments having sizes between 50-600 basepairs, in 50-basepair increments, was prepared as in Example 1. This mixture was added to the FAM-labeled pBR322 fragments in a DNA ratio of about 4:1.

A slab agarose gel containing 2% agarose was prepared in the above Tris-borate buffer by standard methods (Maniatis) to slab dimensions of 4 mm thick, 20 cm width, and 16 cm length in the submarine gel tray provided with the 362 GENE SCANNER (Applied Biosystems, Foster City, CA).

To a gel well was added 2.5 μ L of the combined pBR322/strand-pair fragment mixture, diluted 1:1 with 2 \times Agarose Loading Buffer (containing 50 mg/mL Ficoll 400-DL, 8.3 mg/mL blue dextran, and 1.67 mg/mL dextran sulfate, all obtained from Sigma Chemical Corporation, St. Louis, MO). Electrophoresis was carried out in a Model 362 GENE SCANNER (Applied Biosystems, Inc, Foster City, CA), using a standard Tris-borate buffer as the running buffer. Electrophoretic voltage was 100 V, 80 ma, 8 W, at a temperature of about 25°C. The peaks were monitored by a laser fluorometer, as described in Example 3. The electropherogram generated from the sample is shown in Figure 9, where the pBR322 fragments (FAM label) are shown in the upper trace, and the strand-pair-fragments (ROX label) are shown in the lower trace. The peak positions (electrophoresis times) of the pBR322 were used to establish a plot of basepair number as a function of electrophoresis time. This plot was then used to determine the sizes of the ROX-labeled fragments. The sizes determined for the ROX-labeled fragment peaks are shown in Table 3 below. The calculated size is within 5 basepairs of the correct value

SUBSTITUTE SHEET

36

for every fragment except the smallest one listed (250 basepairs), which lies outside of the size range accurately defined by the pBR322 fragments.

5

10

15

Table 3	
Fragment Size	Calculated Size
250	232
300	297
350	346
400	396
450	446
500	495
550	546
600	595

Example 6

20

Restriction Digest Analysis on Agarose Gels

The FAM JOE, and TAMRA-labeled phage lambda restriction fragments from Example 3 were combined with ROX-labeled fragments of 50-1000 basepairs prepared according to the present invention (Example 1). The mixture was diluted 1:1 with 2x Agarose Loading Buffer and 5 μ L aliquot was separated on a 2% agarose gel as described in Example 5. Figure 10 shows the electropherograms for the lambda restriction fragments (upper panels) and the ROX-labeled standard fragments (bottom panel). The Model 362 GENE SCANNER Analysis Software (Applied Biosystems, Foster City, CA) used the ROX-labeled fragment positions to establish the relationship between electrophoresis time and fragment size, and then calculated sizes for each of the observed lambda restriction fragments. In another gel lane, the

SUBSTITUTE SHEET

37

lambda fragments were analyzed by comparison to ROX-labeled restriction fragments (GENESCAN-2500™ ROX, Applied Biosystems, Foster City, CA).

5	Table 4 Fragment Size		
10	Selected Size Ladder Found	Hind III/Hae II Expected	GENESCAN-2500 Found
15	131 152 233 371 624 568 875 937	136 159 229 371 615 697 872 948	134 153 233 369 629 570 908 980
	5.65	chi squared	7.62
20	Selected Size Ladder Found	Bam HI/Hae III Expected	GENESCAN-2500 Found
25	123 203 288 340 439 459 806	124 203 288 339 439 453 816	125 204 287 338 439 459 829
	0.57	chi squared	1.05
30	Selected Size Ladder Found	Eco RI/Hae III Expected	GENESCAN-2500 Found
35	119 148 259 309 545 730 1000	119 147 256 308 537 733 1038	122 150 258 308 546 745 1058
	2.26	chi squared	1.31

SUBSTITUTE SHEET

Example 7Large-Scale Preparation of a Size LadderStrand-Pair Mixture

The same amplification primers were used as in Example 1. The 10× amplification buffer was similar, except that it contained 15 mM magnesium chloride. The M13mp18(+) template (Promega Corporation, Madison, WI) was diluted to 25 µg/mL with sterile water. A dNTP mix was prepared containing dCTP, dGTP, and dTTP at 2.5 mM, plus dATP at 1.5 mM. Each fragment was prepared by combining water (835 µL), 10× buffer (100 µL), template (10 µL, 250 ng), ROX-labeled forward primer (20 µL, 200 pmol), reverse primer (10 µL, 200 pmol), dNTP mix (20 µL, reaction concentration 50 µM dCTP, dGTP, dTTP and 30 µM dATP), and AmpliTaq® (Perkin Elmer Cetus, Norwalk, CT; 5 µL, 25 units). After mixing, each solution was divided into five 200 µL aliquots in five separate 0.5 mL microcentrifuge tubes. Each aliquot was covered with mineral oil (75 µL). The tubes were placed in a preheated (95°C) DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT) and 30 cycles consisting of double strand denaturation (95°C/30 sec), primer annealing (55°C/30 sec), and polymerase-mediated primer extension (70°C/30 sec) were executed.

Following thermal cycling, the five aliquots were recombined with 1 mL of 1× TE buffer (10 Tris-HCl 1 mM EDTA, pH 8.0) in a Centricon-100 filter (Amicon Division of W.R. Grace & CO., Beverly, MA). The mixture was filtered by centrifugation at 5000 RPM (approximately 3000× g) for 10 minutes at 4°C. The products were recovered in approximately 50 µL of TE buffer by inverting the filter and spinning at 5000 RPM for 2 minutes. After dilution to 1.0 mL with TE buffer, the DNA yield was determined by measurement of A_{260} in a 1.0 cm cuvette. The yields of all frag-

ments from 150-1000 basepairs are shown in Table 5. It is evident that fragments smaller than 300 basepairs were recovered with reduced efficiency on the Centricon-100 filter.

5

Table 5					
Large Scale Fragment Synthesis					
	Fragment Size, bp	ODU Recovered	ODU/nmol (duplex)	Yield, pmol	$\mu\text{L}/100$ fmol
10	150	0.036	2.4	15.0	6.7
	200	0.030	3.2	9.4	10.6
	250	0.060	4.0	15.0	6.7
	300	0.203	4.8	42.3	2.4
	350	0.217	5.6	38.8	2.6
15	400	0.302	6.4	47.2	2.1
	450	0.362	7.2	50.3	2.0
	500	0.422	8.0	52.8	1.9
	550	0.357	8.8	40.6	2.5
	600	0.393	9.6	41.0	2.4
20	650	0.357	10.4	34.3	2.9
	700	0.363	11.2	32.4	4.1
	750	0.372	12.0	31.0	3.2
	800	0.330	12.8	25.8	3.9
	850	0.299	13.6	22.0	4.5
25	900	0.314	14.4	21.8	4.6
	950	0.343	15.2	22.6	4.4
	1000	0.426	16.0	26.6	3.8

Strand pair mixtures were prepared by combining aliquots of all the fragment sizes listed in Table 5, as well as synthetic double stranded 50 and 100 basepair

fragments, in the proportions indicated in the last column of the table, to give equal molar ratios of the fragments. The fragments were then precipitated by addition of sodium acetate to 0.3 M, followed by 2.5 volumes of ethanol, as
5 described in Examples 1 and 3. After centrifugation and removal of the supernatant, the strand-pair mixture was resuspended in the desired amount of TE buffer, from which individual aliquots containing 5-10 fmol of each fragment could be removed for analysis.

10 Although the invention has been described with respect to preferred compositions and methods, it will be apparent that various modifications can be made without departing from the invention.

IT IS CLAIMED:

1. A polynucleotide strand-pair mixture for use in calibrating the basepair numbers of one or more analytes
5 fractionated by electrophoresis, comprising
a plurality of polynucleotide strand pairs, each pair having one strand which has a selected number of polynucleotide bases and a detectable reporter in its 5'-end region and a complementary strand which lacks said reporter, where
10 the concentration of said complementary strand may be different from the concentration of the corresponding reporter-containing strand, and where the selected number of bases in each pair's reporter-containing strand is different for each pair in the mixture.
15
2. The strand-pair mixture of claim 1, wherein the reporter is a radiolabeled atom in a nucleotide in the 5'-end region of the reporter-containing strand in each strand pair.
20
3. The strand-pair mixture of claim 1, wherein the reporter is a fluorescence reporter carried on the 5'-end nucleotide of the reporter-containing strand in each strand pair.
25
4. The strand-pair mixture of claim 1, wherein the selected number of bases in the reporter-containing strands in the strand pairs differ from one another by a multiple of a selected number of bases.
30
5. The fragment mixture of claim 4, wherein the strand pairs differ in size by a multiple of about 40-60 bases.

6. The fragment mixture of claim 4, for use in high-resolution polyacrylamide gel electrophoresis in the presence of a denaturant agent, wherein the strand pairs have selected sizes less than about 1,200 bases.

5

7. The fragment mixture of claim 1, wherein each successively larger reporter-containing strand in the mixture contains the same basepair sequence as the next-smaller reporter-containing strand, plus a 3'-end sequence
10 of at least about 10 bases.

8. The fragment mixture of claim 7, wherein each successively larger reporter-containing strand differs from the next-smaller strand by substantially the same number of
15 bases.

9. The fragment mixture of claim 1, for use in detecting a small base number mutation in a genomic segment having a given number of basepairs, wherein the mixture
20 contains first and second fragments whose reporter-containing strands differ from one another by a selected number of bases, and are shorter and longer, respectively, than the genomic segment in the absence of a mutation.

25 10. A method of producing a polynucleotide fragment mixture for use in calibrating the basepair numbers of one or more polynucleotide analytes fractionated by electrophoresis, comprising

identifying a segment of a DNA template having a
30 selected number of basepairs between about 25-2,000 basepairs,

reacting the segment-containing template with reporter-labeled and unlabeled single-stranded primers which are complementary to 3'-end regions of opposite strands of the identified segment, when the segment is in a double-stranded form, under reaction conditions which produce multiple rounds of primer-mediated duplex DNA replication, by said reacting, producing a duplex DNA fragment having the basepair composition of said segment, and one strand only which includes the reporter-labeled primer, and repeating said identifying and reacting steps by (i) identifying a segment of a DNA template containing a selected number of basepairs which is different from that of an already-selected segment, (ii) reacting the DNA template containing the just-identified segment with reporter-labeled and unlabeled single-stranded primers which are complementary to 3'-end regions of opposite strands of the just-identified segment, when the segment is in double-stranded form, under reaction conditions which produce multiple rounds of primer-mediated duplex DNA replication, thereby producing a duplex DNA fragment having the basepair composition of the just-identified segment, and one strand only which includes the reporter label, until a desired number of different-length fragments are produced.

25

11. The method of claim 10, wherein the templates containing the selected-length segments are single-stranded DNA templates.

30

12. The method of claim 10, wherein the different selected-length segments are contained in a single template, and have an overlapping segment end region which is common to each of the segments.

13. The method of claim 12, wherein the labeled primer is complementary to said common-sequence segment 3'-end region.

5 14. The method of claim 13, wherein the fragments are produced in a single reaction mixture containing said template, the labeled primer, and a series of unlabeled primers which are complementary to the 3' ends of the selected-length segment strands which are opposite to the
10 common-sequence end region.

15 15. The method of claim 10, for use in determining the amounts of one or more of such polynucleotide analytes fractionated by electrophoresis, wherein the fragments are produced individually and combined in known concentrations to produce the mixture.

20 16. The method of claim 15, wherein the different selected-length segments are contained in a single template, and have an overlapping segment end region which is common to each of the segments, and the labeled primer is homologous to said common-sequence segment end region.

25 17. The method of claim 10, for use in calibrating the basepair numbers of one or more polynucleotide analytes fractionated by polyacrylamide gel electrophoresis in the presence of a denaturant agent, wherein the selected segments contain less than about 1,200 bases, and differ from one another in number of bases by a multiple of about
30 40-60 bases.

18. The method of claim 10, wherein the reporter is a fluorescent reporter carried on the 5'-end nucleotide of the reporter-containing primer.

5 19. In a method for genomic mapping in which individual library genomic fragments are digested by restriction endonucleases, to produce digest subfragments having sizes in the size range between about 50-2,000 basepairs, and the subfragments are (i) labeled in at least one strand
10 with a fluorescent reporter, (ii) fractionated by high-resolution electrophoresis in a polyacrylamide matrix, and (iii) compared according to size with subfragments derived from other library genomic fragments, to determine fragments which have overlapping regions, an improvement for
15 enhancing the resolution of subfragment size comprising fractionating the subfragments in the presence of a polynucleotide mixture composed of a plurality of polynucleotide strand pairs, each pair having one strand which has a selected number of polynucleotide bases and a
20 detectable fluorescence reporter in its 5'-end region and a complementary strand which lacks said reporter, where the selected number of bases in each fragment's reporter-containing strand is between about 30-2,000 and is different for each fragment in the mixture, and
25 determining the size of the subfragments, to a resolution of 1-2 bases, from their relative migration rates in the acrylamide matrix with the known sizes of the reporter-containing strand-pair polynucleotide strands.

30

20. The improvement of claim 19, wherein the subfragments and polynucleotide mixture are fractionated in single-stranded form on a polyacrylamide gel in the presence of a denaturing agent, and the size of the subfragments is determined to a 1-base resolution.

21. The improvement of claim 19, wherein the subfragments and polynucleotide mixture are fractionated in double-stranded form on an agarose, polyacrylamide, or other gel.

22. The improvement of claim 19, wherein the fluorescent probe carried on the digest subfragments is distinguishable from the fluorescent probe carried on the fragment polynucleotide strand pairs.

23. In a dideoxy polynucleotide sequencing method in which various-length fragments which terminate at one of four dideoxynucleotide bases are fractionated by high-resolution electrophoresis in the presence of a denaturing agent, and the sequence of the polynucleotide is determined from the basepair number determined for each fragment, an improvement for increasing the resolution of basepair number determination comprising

fractionating the various-length fragments in the presence of a polynucleotide mixture composed of a plurality of polynucleotide strand pairs, each pair having one strand which has a selected number of polynucleotide bases and a detectable fluorescence reporter in its 5'-end region and a complementary strand which lacks said reporter, where the selected number of bases in each fragment's reporter-containing strand is between about 30-2,000 and is different for each fragment in the mixture, and

determining the size of the various-length fragments, to a resolution of 1 base, from their relative migration rates in the acrylamide matrix with the known sizes of the reporter-containing strand-pair polynucleotide strands.

5

24. The improved method of claim 23, wherein the various-length fragments have a distinctive fluorescence reporter for each dideoxynucleotide, and the various length fragments with the different reporters and the reporter-labeled strand pairs are co-electrophoresed.

25. The improved method of claim 23, wherein the various-length fragments which terminate at a common dideoxynucleotide and the reporter-labeled pair strands are co-electrophoresed.

26. A method of quantitating the amount of one or more nucleic acid fragments fractionated by electrophoresis comprising,

20 mixing the fragments with a polynucleotide mixture composed of a plurality of polynucleotide strand pairs, each pair having one strand which has a selected number of polynucleotide bases and a detectable fluorescence reporter in its 5'-end region and a complementary strand which lacks

25 said reporter, where the selected number of bases is different for each fragment in the mixture, and the concentration of each strand pair in the mixture is known, fractionating the fragments and polynucleotide mixture,

30 determining the relationship between fragment concentration and total fluorescence emission, as a function of number of bases, for strand pairs polynucleotides whose concentrations are known, and

48

using said relationship to determine the concentration of analyte fragments, according to total fluorescence emission of the fragments and number of bases in the fragments.

5

27. The method of claim 26, wherein the fragments and polynucleotides in the mixture are fractionated on polyacrylamide gel in the presence of a denaturant agent.

SUBSTITUTE SHEET

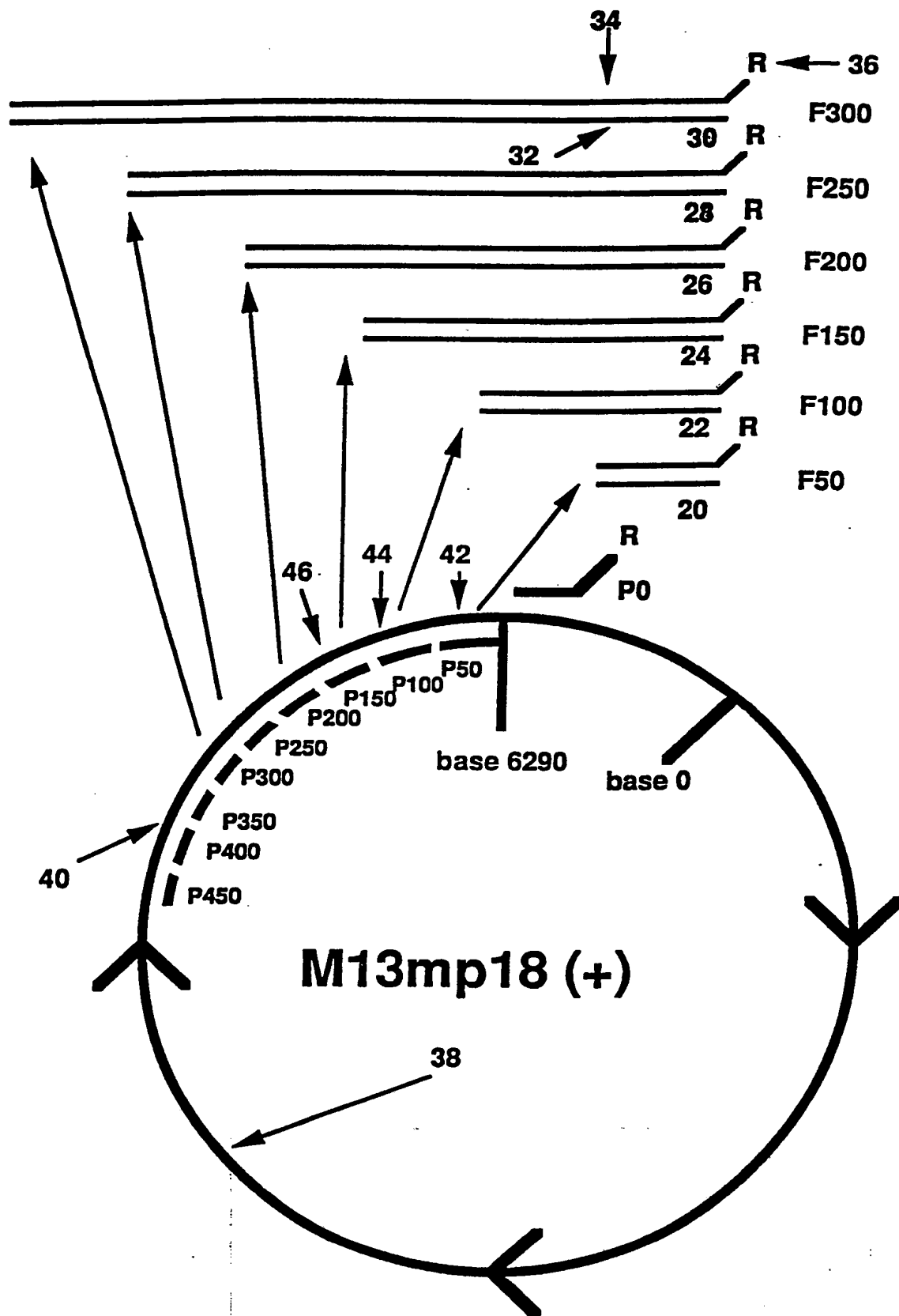


Fig. 1

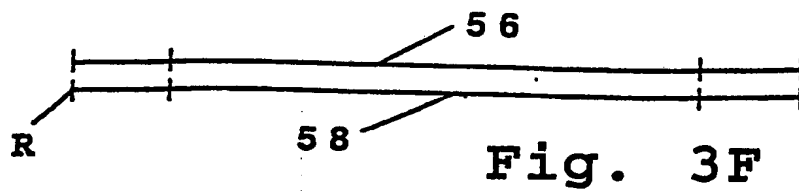
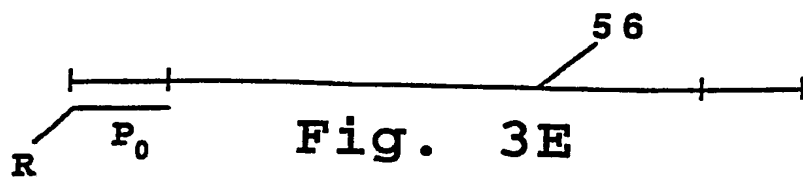
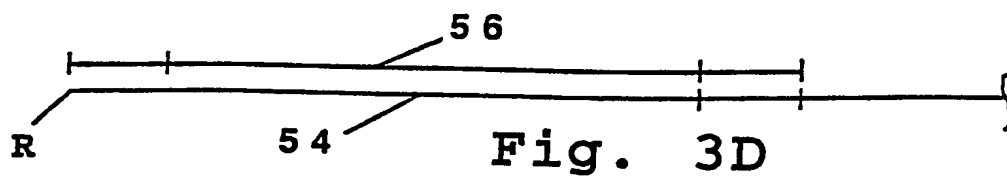
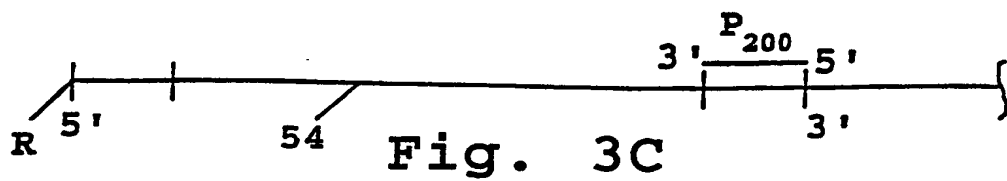
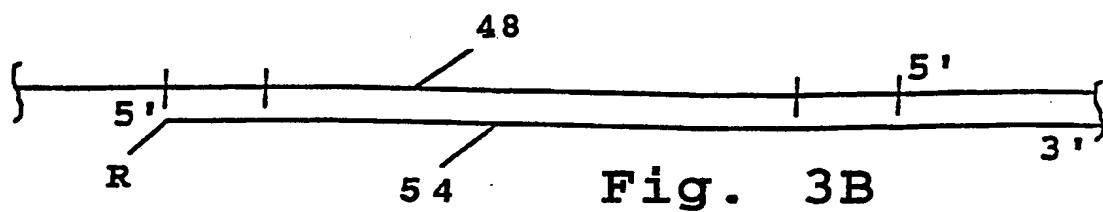
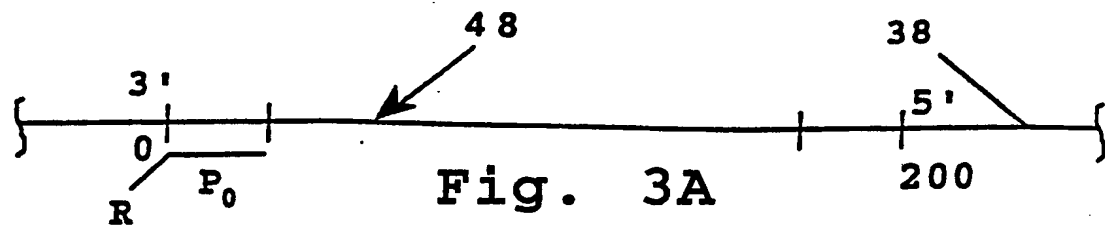
2/14

3'- P₀

ACATTTTGCT GCCGGTCACG GTTCGAACGT ACGGACGTCC AGCTGAGATC P₅₀
TCCTAGGGGC CCATGGCTCG AGCTTAAGCA TTAGTACCAG TATCGACAAA P₁₀₀
GGACACACTT TAACAATAGG CGAGTGTTAA GGTGTGTTGT ATGCTCGGCC P₁₅₀
TTCGTATTTT ACATTTTCGA CCCCACGGAT TACTCACTCG ATTGAGTGTA P₂₀₀
ATTAACGCAA CGCGAGTGAC GGGCGAAAGG TCAGCCCTTT GGACAGCACG P₂₅₀
GTCGACGTAA TTACTT~~A~~.GCC GGTTCGCGCG CCCTCTCCGC CAAACGCATA P₃₀₀
ACCCGCGGTC CCACCAAAAA GAAAAGTGGT CGCTCTGCCC GTTGTCGACT P₃₅₀
AACGGGAAGT GGCGGACCGG GACTCTCTCA ACGTCGGTTCG CCAGGTGCCA P₄₀₀
CCAAACGGGG TCGTCCGCTT TTAGGACAAA CTACCACCAA GGCTTTAGCC P₄₅₀
GTTTTAGGGA ATATTTAGTT TTCTTATCGG GCTCTATCCC AACTCACAAC P₅₀₀
AAGGTCAAAC CTTGTTCTCA GGTGATAATT TCTTGACCT GAGGTTCAG P₅₅₀
TTTCCCGCTT TTTGGCAGAT AGTCCCGCTA CCGGGTGATG CACTTGGTAG P₆₀₀
TGGGTTTAGT TCAAAAACC CCAGCTCCAC GGCATTTTCGT GATTAGCCT P₆₅₀
TGGGATTTC CTCGGGGGCT AAATCTCGAA CTGCCCCCTT CGGCCGCTTG P₇₀₀
CACCGCTCTT TCCTTCCCTT CTTTCGCTTT CCTCGCCCGC GATCCCGCGA P₇₅₀
CCGTTACAT CGCCAGTGCG ACGCGCATTG GTGGTGTGGG CGGCGCGAAT P₈₀₀
TACGCGGCGA TGTCCCGCGC ATGATACCAA CGAAACTGCT CGTGCAATAT P₈₅₀
GCACGAAAG AGCAACCTTA GTCTCGCCCT CGATTTGTCC TCCGGCTAAT P₉₀₀
TTCCCTAAAA TCTGTCCTTG CCATGCGGTC TTAGAACTCT TCACAAAAAT P₉₅₀
ATTAGTCACT CCGGTGGCTC ATTTTCTCAG ACAGGTAGTG CGTTTAATTG P₁₀₀₀
GCAACATCGT TATGAAGAAA CTAATCATTA TTGTAGTGAA CGCACTCATC-5'

Fig. 2

3/14



4/14

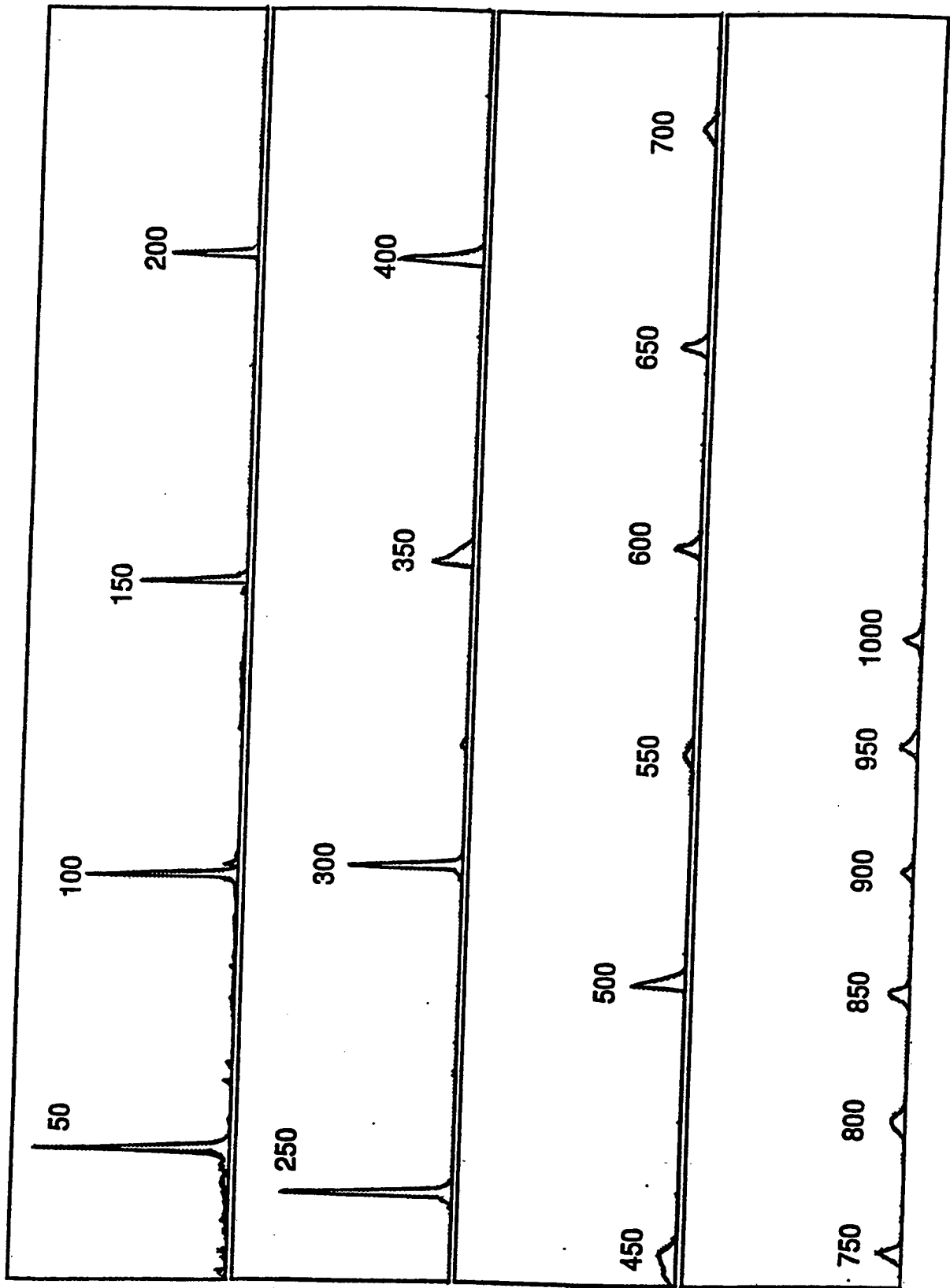
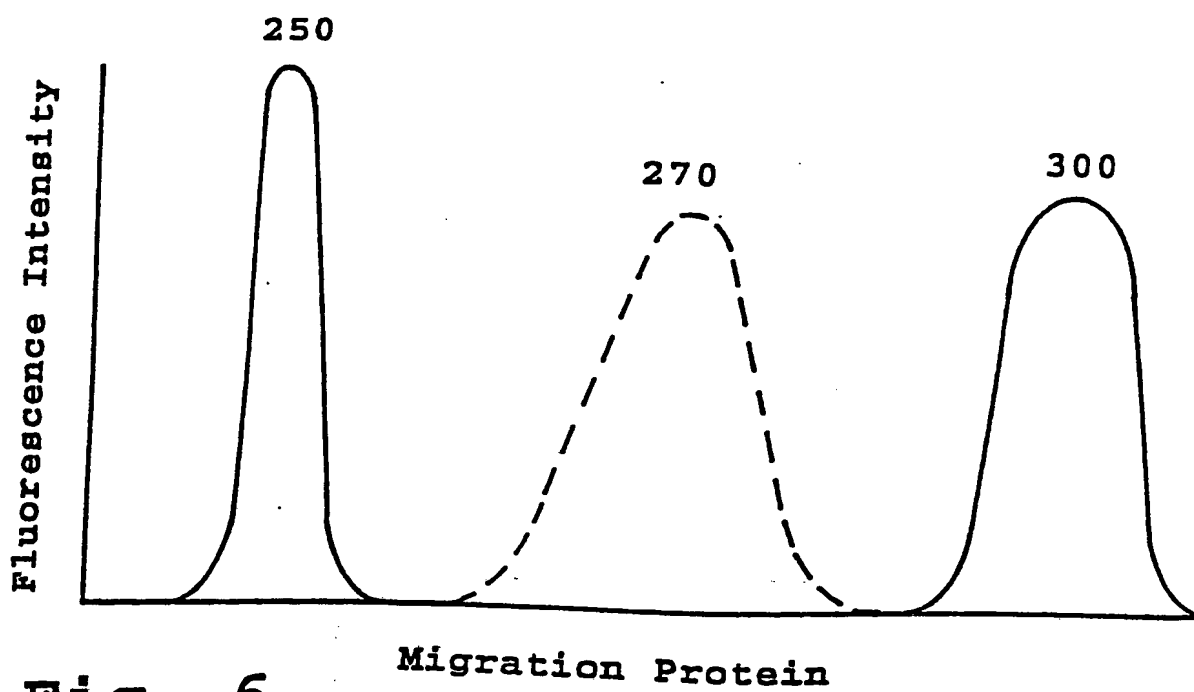
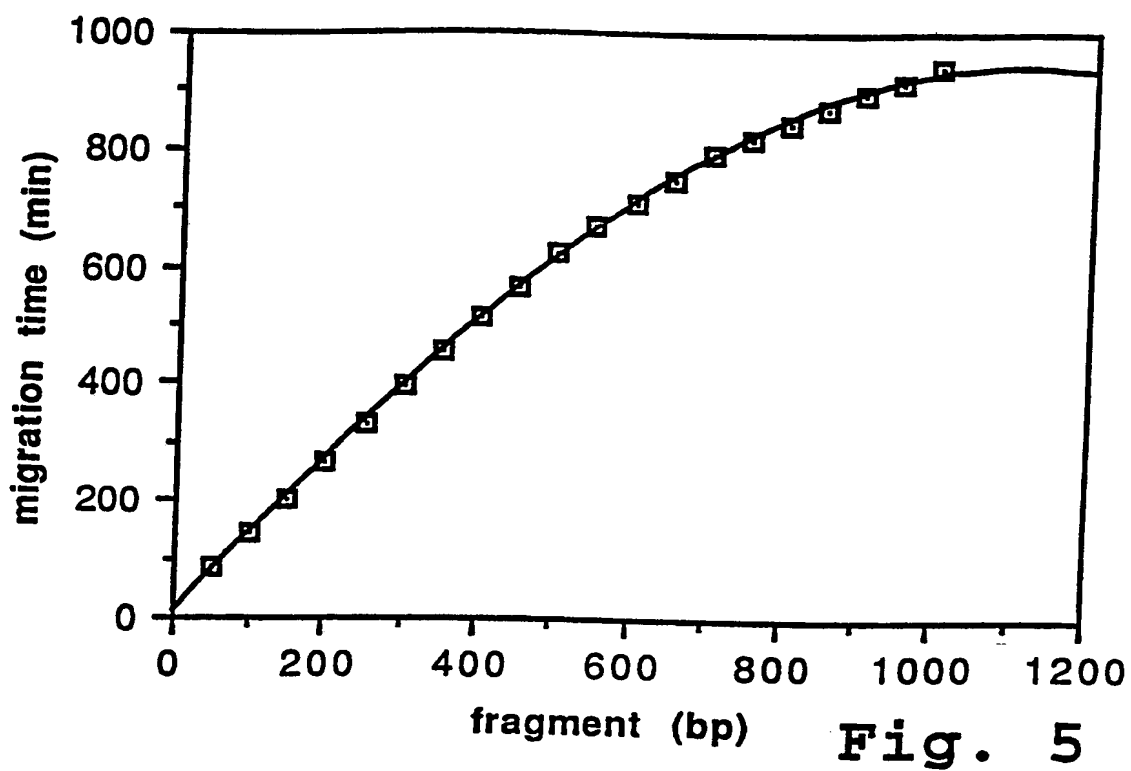


Fig. 4

5/14



6/14

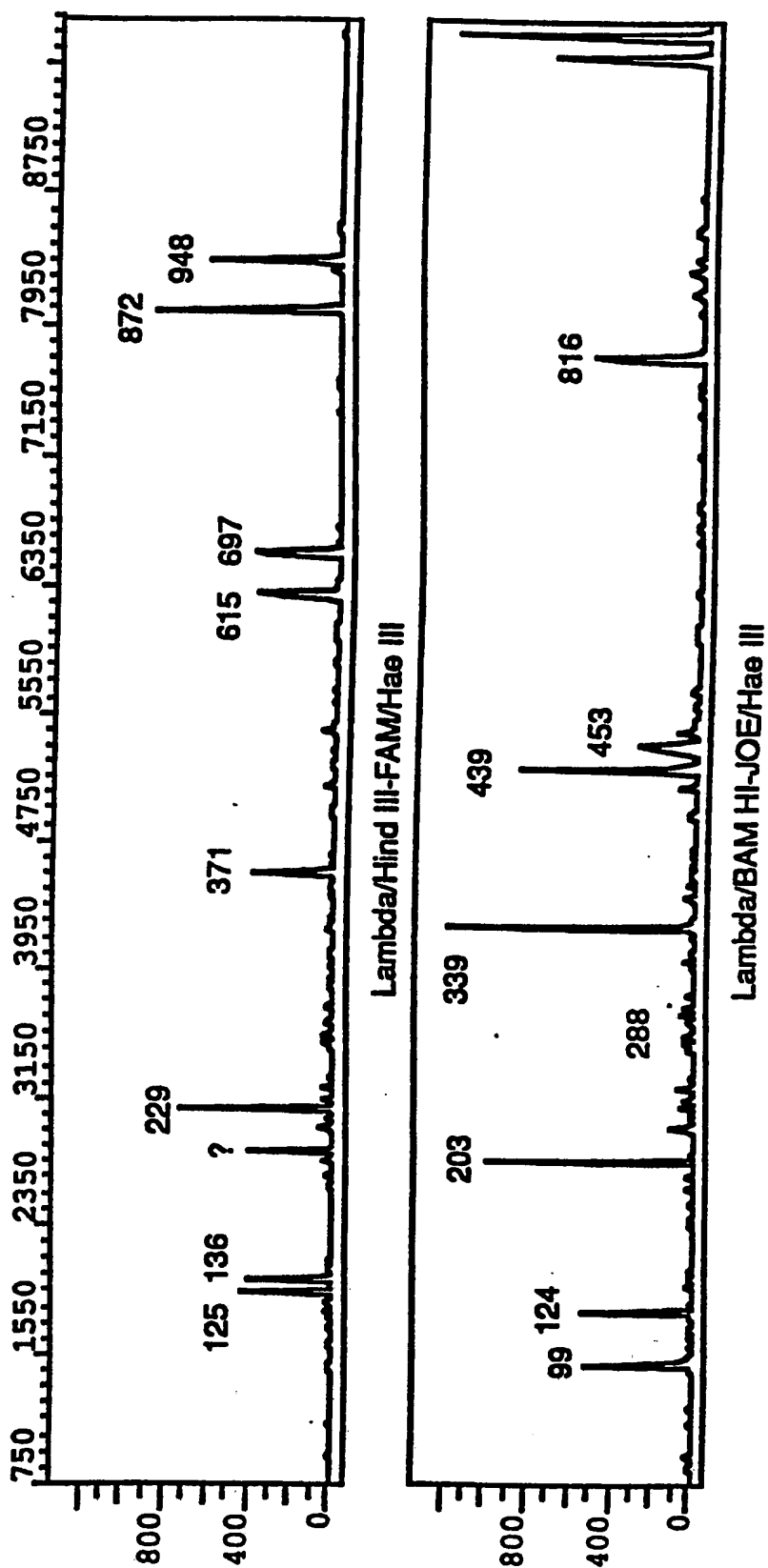


Fig. 7

7/14

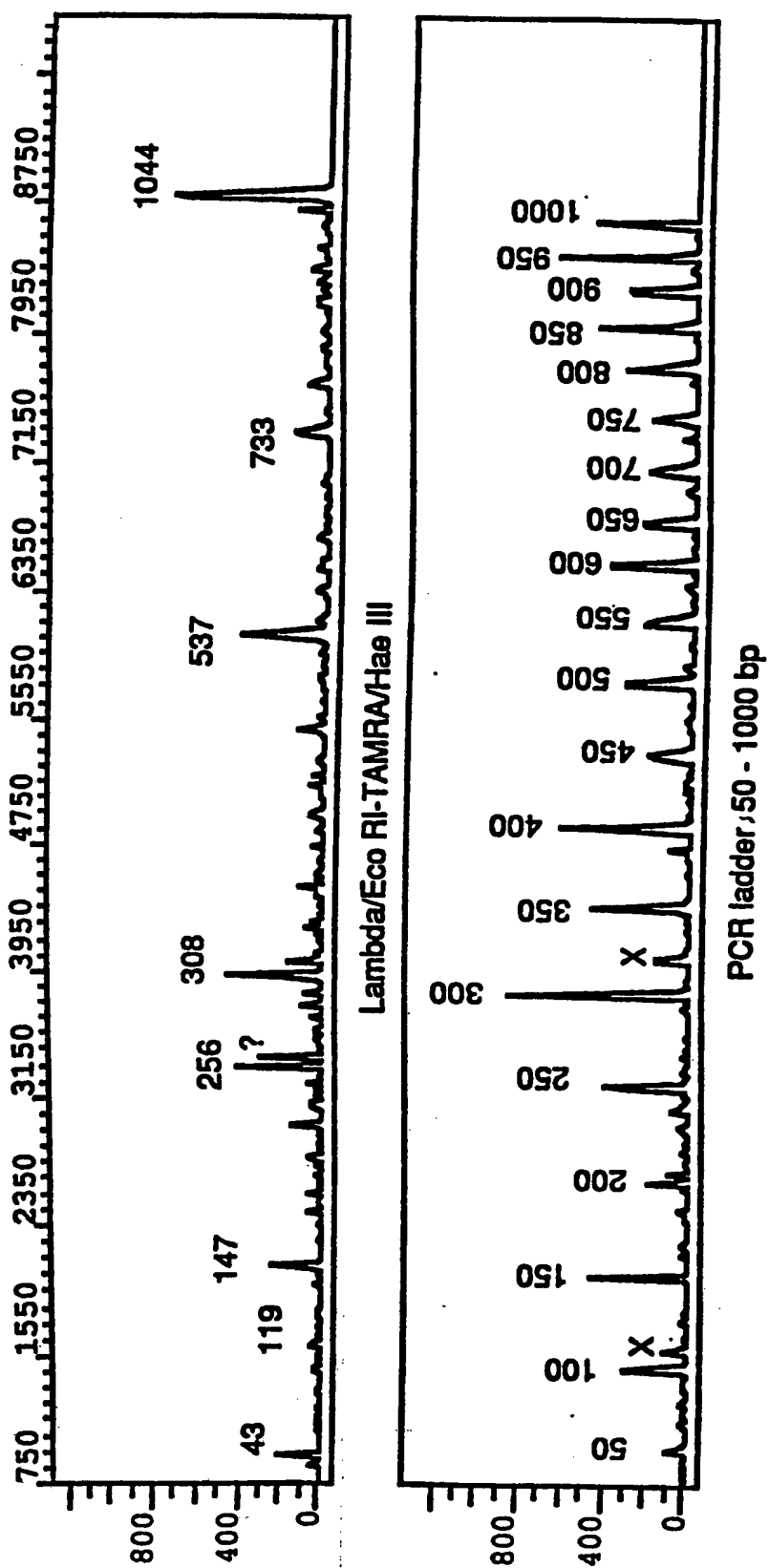


Fig. 7 (con't)

8/14

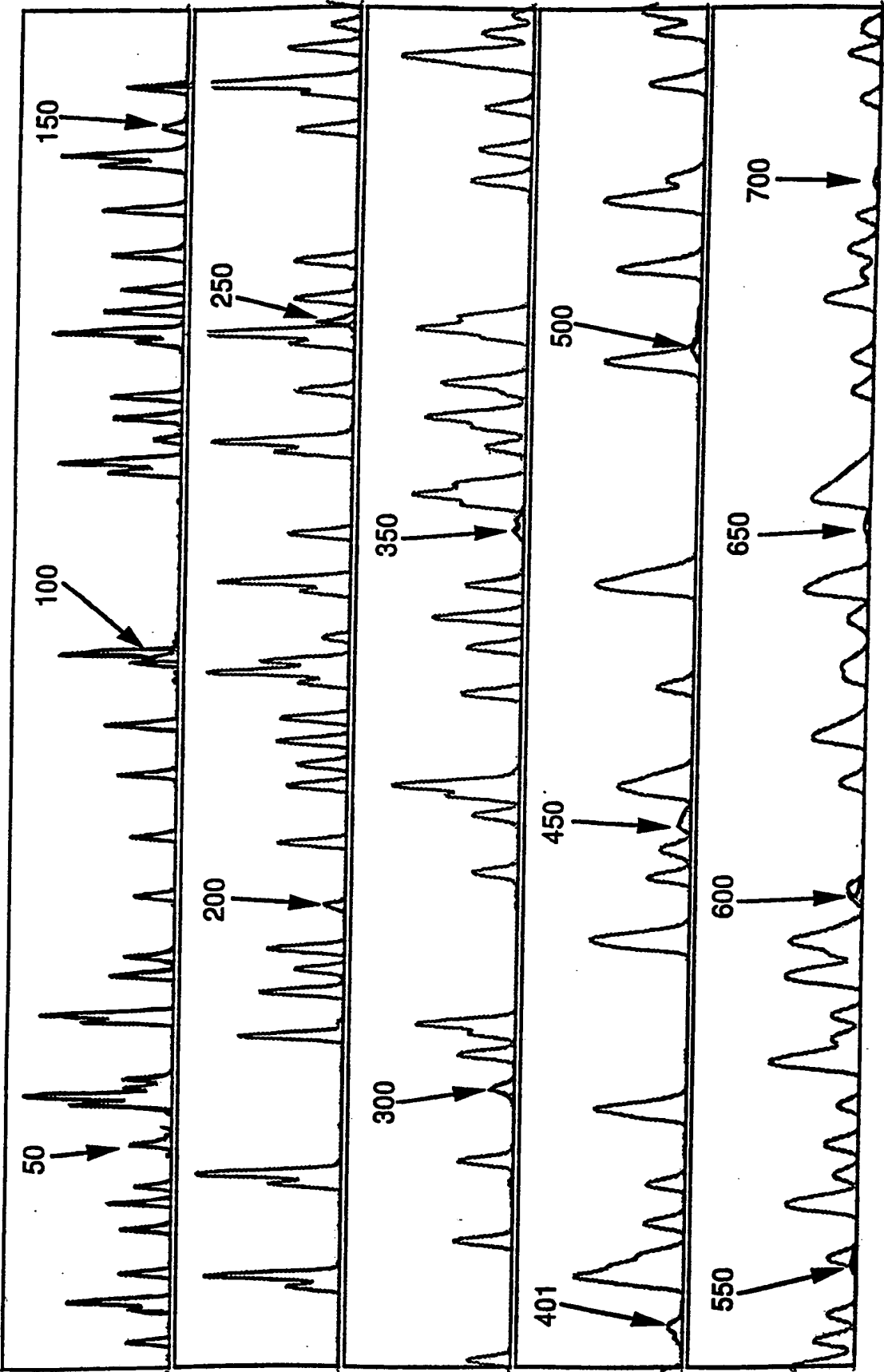


Fig. 8A

9/14

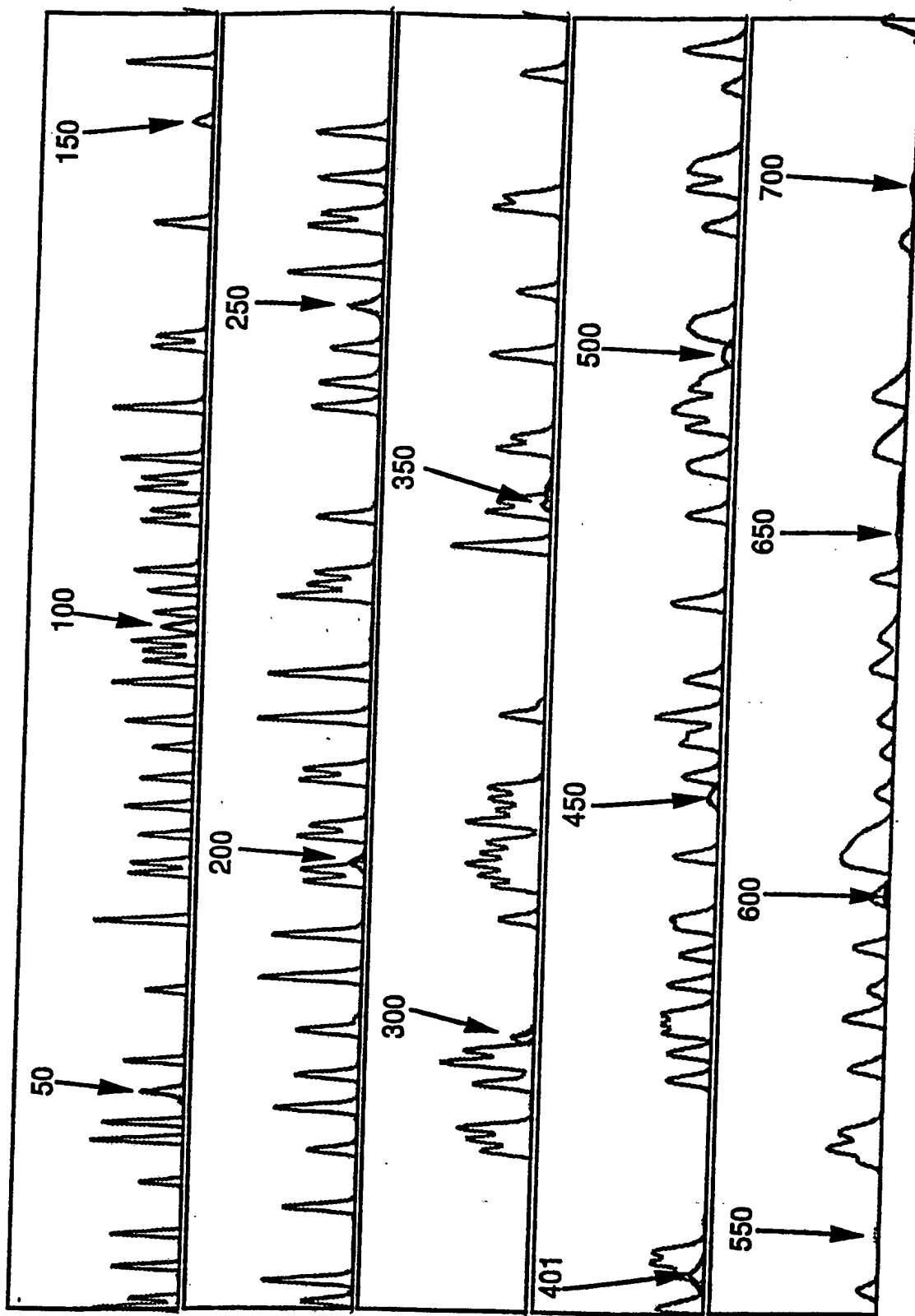


Fig. 8B

10/14

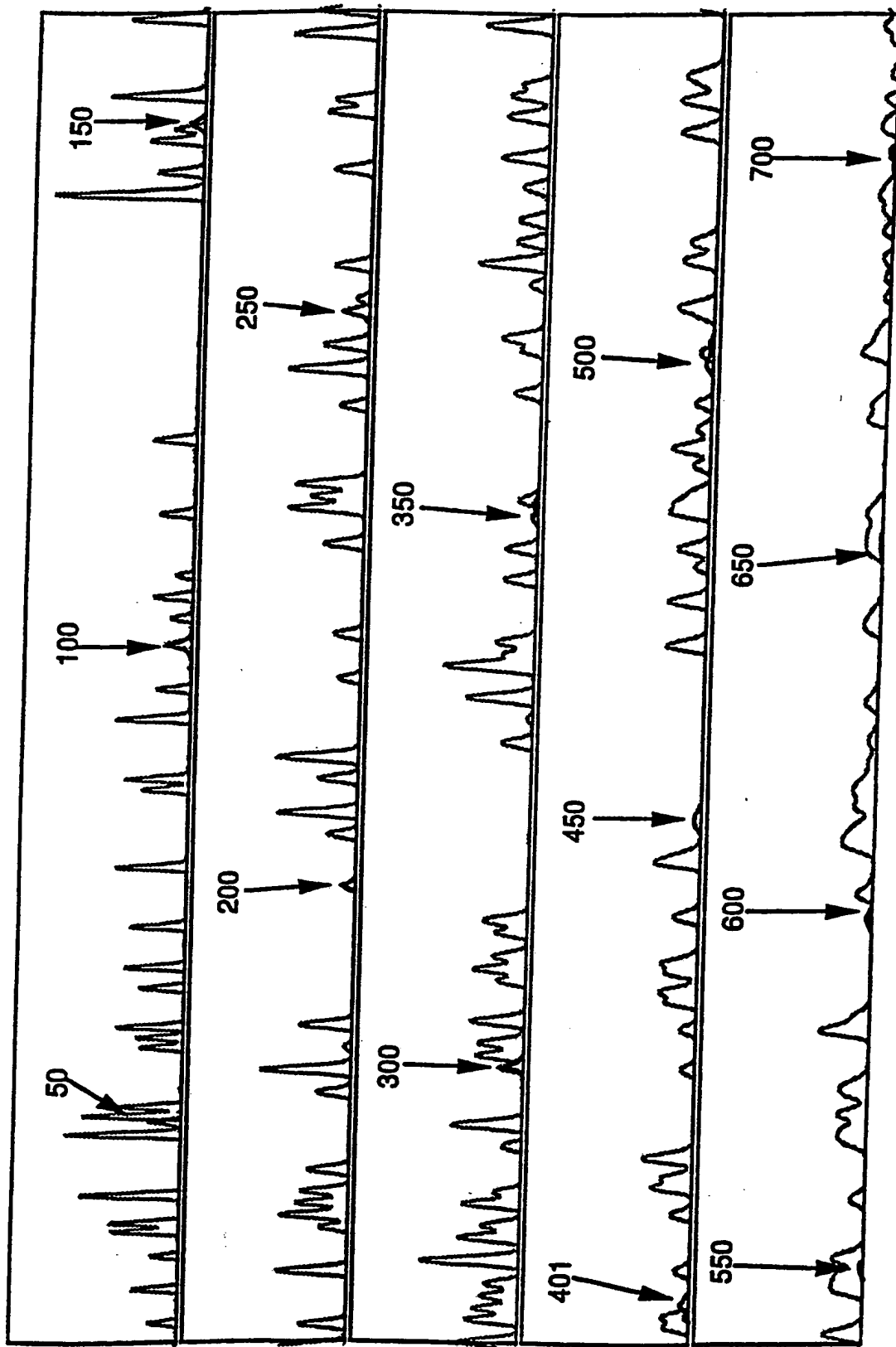


Fig. 8C

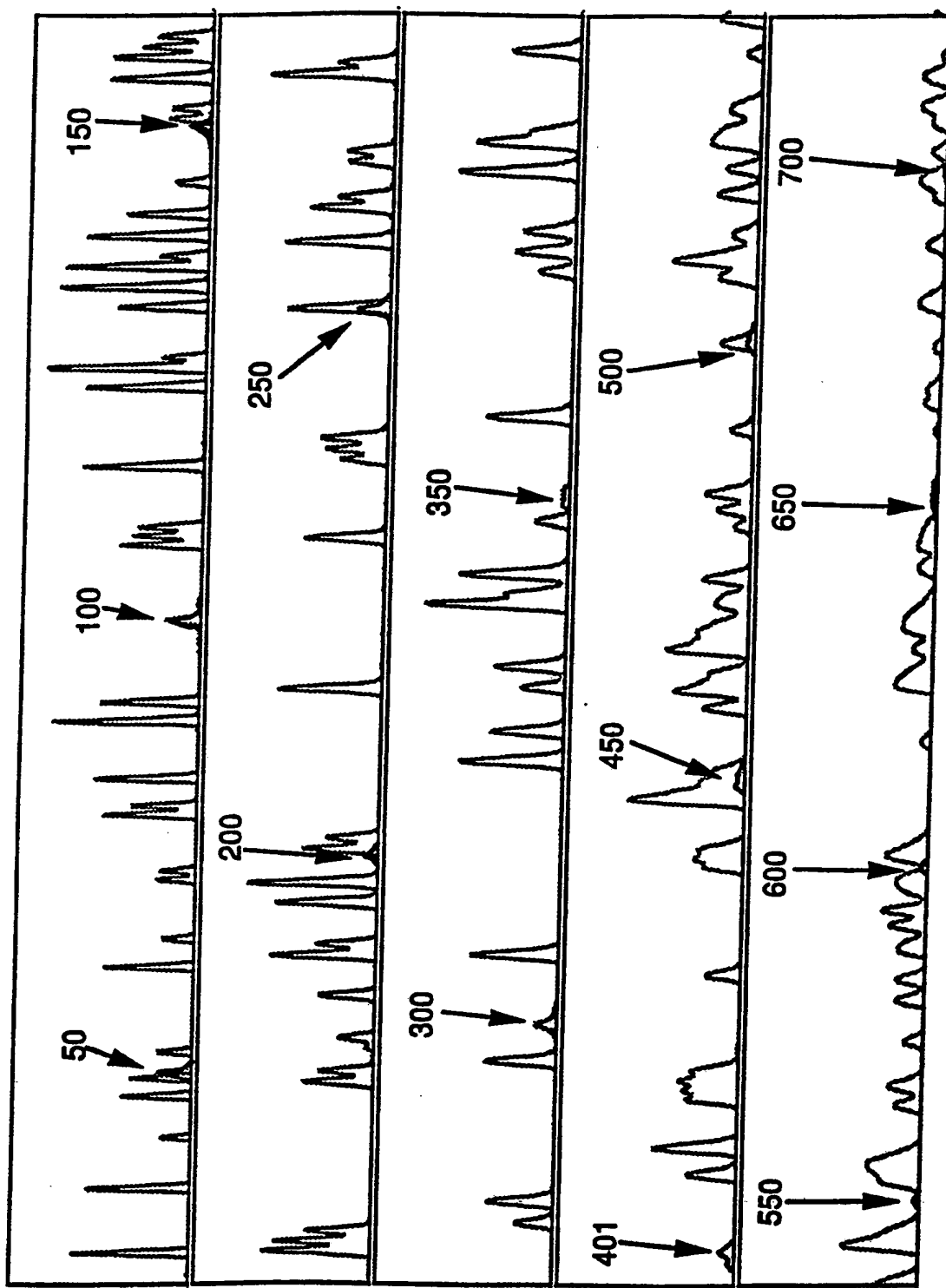
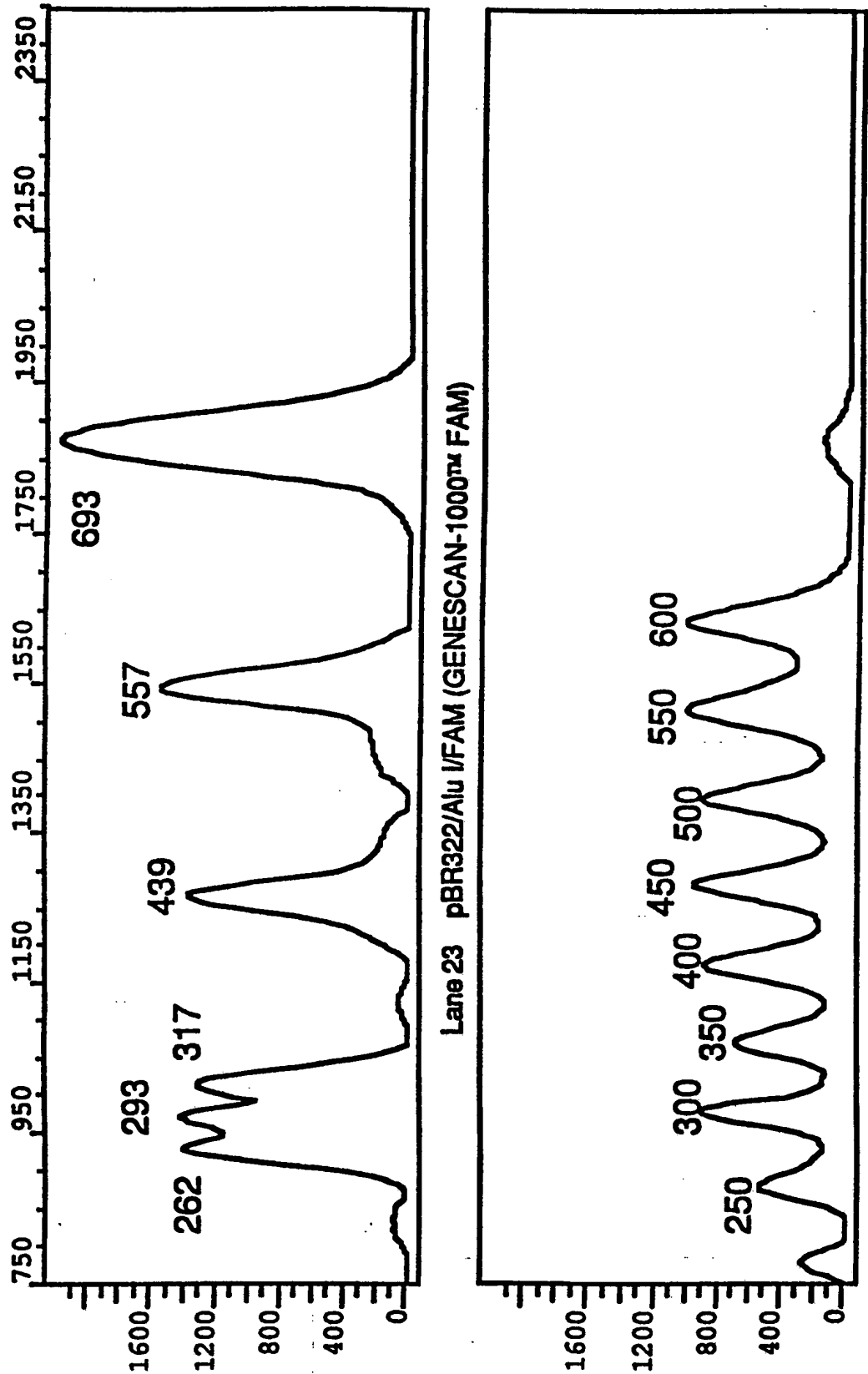


Fig. 8D

12/14



Lane 23 ROX-labeled fragments, 50-600 basepairs

Fig. 9

13/14

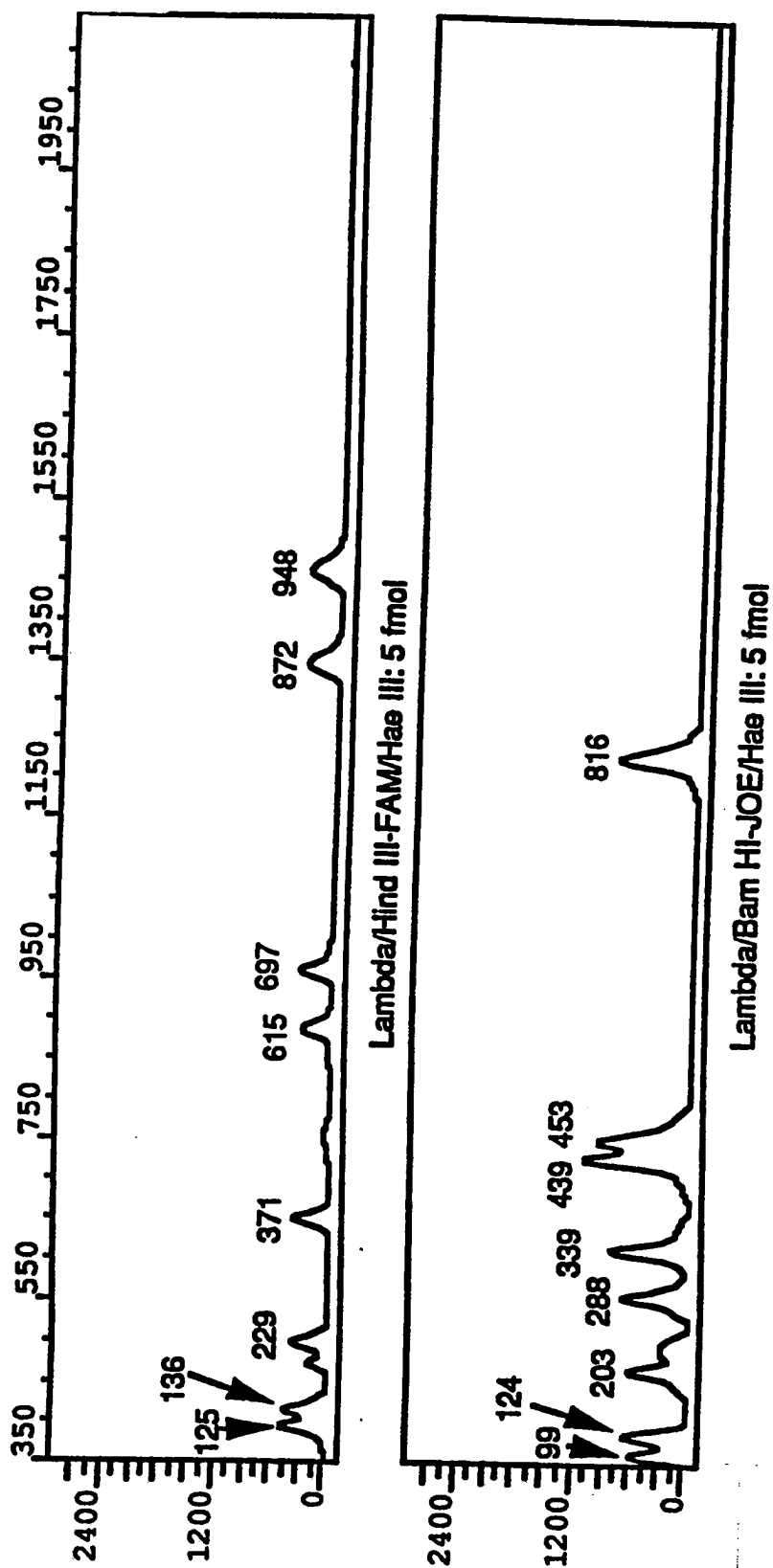


Fig. 10

14/14

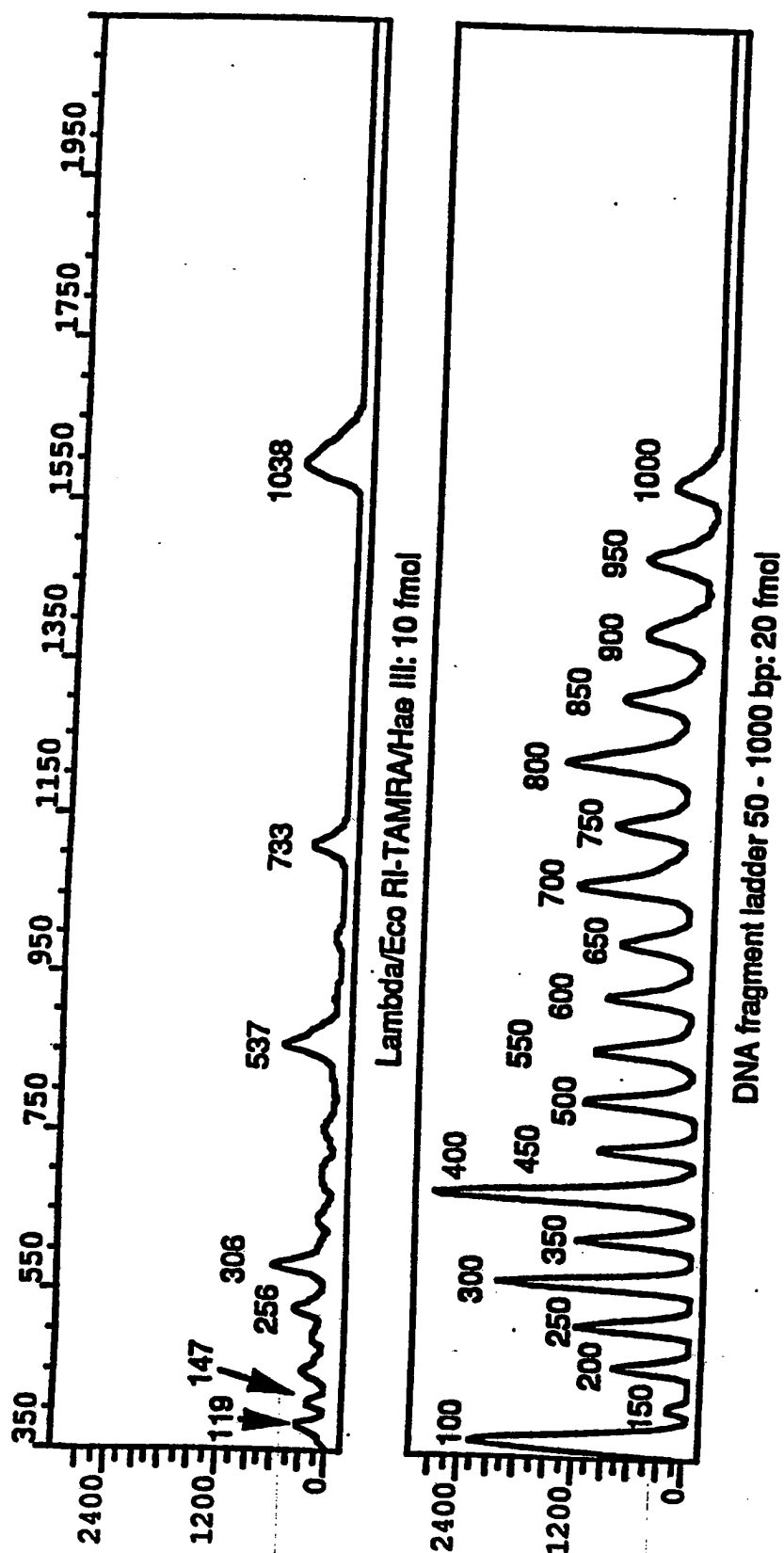


Fig. 10 (con't)

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 93/00100

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12Q1/68; G01N27/26

II. FIELDS SEARCHEDMinimum Documentation Searched⁷

Classification System

Classification Symbols

Int.Cl. 5

C12Q

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched⁸**III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹**

Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	EP,A,0 393 743 (EASTMAN KODAK CO.) 24 October 1990 see the whole document ----	1-18
Y	WO,A,9 118 095 (THE BOARD OF REGENTS OF THE UNIVERSITY OF TEXAS) 28 November 1991 see the whole document ----	1-18
A	EP,A,0 359 225 (DU PONT DE NEMOURS AND CO.) 21 March 1990 see page 13, line 14 - line 40 ----	1-18
A	EP,A,0 388 053 (CHEMBIOMED LMT.) 19 September 1990 see the whole document ---- -/--	19-22

⁹ Special categories of cited documents :¹⁰^{"A"} document defining the general state of the art which is not
considered to be of particular relevance^{"E"} earlier document but published on or after the international
filing date^{"L"} document which may throw doubts on priority claim(s) or
which is cited to establish the publication date of another
citation or other special reason (as specified)^{"O"} document referring to an oral disclosure, use, exhibition or
other means^{"P"} document published prior to the international filing date but
later than the priority date claimed^{"T"} later document published after the international filing date
or priority date and not in conflict with the application but
cited to understand the principle or theory underlying the
invention^{"X"} document of particular relevance; the claimed invention
cannot be considered novel or cannot be considered to
involve an inventive step^{"Y"} document of particular relevance; the claimed invention
cannot be considered to involve an inventive step when the
document is combined with one or more other such docu-
ments, such combination being obvious to a person skilled
in the art.^{"&"} document member of the same patent family**IV. CERTIFICATION**

Date of the Actual Completion of the International Search

21 MAY 1993

Date of Mailing of this International Search Report

10. 06. 93

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

MOLINA GALAN E.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9300100
SA 69161

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

21/05/93

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0393743	24-10-90	CA-A-	2013317	17-10-90
		JP-A-	2299599	11-12-90
WO-A-9118095	28-11-91	US-A-	5162514	10-11-92
		EP-A-	0533750	31-03-93
EP-A-0359225	21-03-90	US-A-	4997928	05-03-91
		JP-A-	2174792	06-07-90
EP-A-0388053	19-09-90	AU-A-	5076490	20-09-90
		CA-A-	2010879	07-09-90
		JP-A-	3043097	25-02-91
EP-A-0233053	19-08-87	US-A-	4855225	08-08-89
		JP-A-	62249049	30-10-87
EP-A-0466479	15-01-92	US-A-	5096557	17-03-92
		JP-A-	4232850	21-08-92
EP-A-0466404	15-01-92	CA-A-	2045794	14-01-92

EP FORM 100/93

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82